9-19-00

Practitioner's Docket No. NEB-135-C **PATENT**

Preliminary Classification:

Proposed Class:

Subclass:

NOTE: "All applicants are requested to include a preliminary classification on newly filed patent applications. The preliminary classification, preferably class and subclass designations, should be identified in the upper right-hand corner of the letter of transmittal accompanying the application papers, for example 'Proposed Class 2, subclass 129.'" M.P.E.P. § 601, 7th ed.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Box Patent Application
Assistant Commissioner for Patents
Washington, D.C. 20231

NEW APPLICATION TRANSMITTAL

Transmitted herewith for filing is the patent application of

Inventor(s): Jay Wayne
Shuang-yong Xu

WARNING: *37 C.F.R. § 1.41(a)(1) points out:*

"(a) A patent is applied for in the name or names of the actual inventor or inventors.

"(1) The inventorship of a nonprovisional application is that inventorship set forth in the oath or declaration as prescribed by § 1.63, except as provided for in § 1.53(d)(4) and § 1.63(d). If an oath or declaration as prescribed by § 1.63 is not filed during the pendency of a nonprovisional application, the inventorship is that inventorship set forth in the application papers filed pursuant to § 1.53(b), unless a petition under this paragraph accompanied by the fee set forth in § 1.17(i) is filed supplying or changing the name or names of the inventor or inventors."

For (title): METHOD FOR CONSTRUCTION OF THERMUS-E. COLI SHUTTLE VECTORS AND IDENTIFICATION OF TWO THERMUS PLASMID REPLICATION ORIGINS

CERTIFICATION UNDER 37 C.F.R. § 1.10*
(Express Mail label number is mandatory.)
(Express Mail certification is optional.)

I hereby certify that this New Application Transmittal and the documents referred to as attached therein are being deposited with the United States Postal Service on this date 18 Sept 2000, in an envelope as "Express Mail Post Office to Addressee," mailing Label Number EK249611825US, addressed to the: Assistant Commissioner for Patents, Washington, D.C. 20231.

Melissa A. Jackson
(type or print name of person mailing paper)

Melissa A. Jackson
Signature of person mailing paper

WARNING: *Certificate of mailing (first class) or facsimile transmission procedures of 37 C.F.R. § 1.8 cannot be used to obtain a date of mailing or transmission for this correspondence.*

***WARNING:** Each paper or fee filed by "Express Mail" **must** have the number of the "Express Mail" mailing label placed thereon prior to mailing. 37 C.F.R. § 1.10(b).*
*"Since the filing of correspondence under § 1.10 without the Express Mail mailing label thereon is an oversight that can be avoided by the exercise of reasonable care, requests for waiver of this requirement will **not** be granted on petition." Notice of Oct. 24, 1996, 60 Fed. Reg. 56,439, at 56,442.*

1. Type of Application

This new application is for a(n)

(check one applicable item below)

☐ Original (nonprovisional)

☐ Design

☐ Plant

WARNING: ***Do not*** *use this transmittal for a completion in the U.S. of an International Application under 35 U.S.C. § 371(c)(4), unless the International Application is being filed as a divisional, continuation or continuation-in-part application.*

WARNING: ***Do not*** *use this transmittal for the filing of a provisional application.*

NOTE: If one of the following 3 items apply, then complete and attach ADDED PAGES FOR NEW APPLICATION TRANSMITTAL WHERE BENEFIT OF A PRIOR U.S. APPLICATION CLAIMED and a NOTIFICATION IN PARENT APPLICATION OF THE FILING OF THIS CONTINUATION APPLICATION.

☐ Divisional.

☒ Continuation.

☐ Continuation-in-part (C-I-P).

2. Benefit of Prior U.S. Application(s) (35 U.S.C. §§ 119(e), 120, or 121)

NOTE: A nonprovisional application may claim an invention disclosed in one or more prior filed copending nonprovisional applications or copending international applications designating the United States of America. In order for a nonprovisional application to claim the benefit of a prior filed copending nonprovisional application or copending international application designating the United States of America, each prior application must name as an inventor at least one inventor named in the later filed nonprovisional application and disclose the named inventor's invention claimed in at least one claim of the later filed nonprovisional application in the manner provided by the first paragraph of 35 U.S.C. § 112. Each prior application must also be:

(i) An international application entitled to a filing date in accordance with PCT Article 11 and designating the United States of America; or

(ii) Complete as set forth in § 1.51(b); or

(iii) Entitled to a filing date as set forth in § 1.53(b) or § 1.53(d) and include the basic filing fee set forth in § 1.16; or

(iv) Entitled to a filing date as set forth in § 1.53(b) and have paid therein the processing and retention fee set forth in § 1.21(l) within the time period set forth in § 1.53(f).

37 C.F.R. § 1.78(a)(1).

NOTE: If the new application being transmitted is a divisional, continuation or a continuation-in-part of a parent case, or where the parent case is an International Application which designated the U.S., or benefit of a prior provisional application is claimed, then check the following item and complete and attach ADDED PAGES FOR NEW APPLICATION TRANSMITTAL WHERE BENEFIT OF PRIOR U.S. APPLICATION(S) CLAIMED.

WARNING: *If an application claims the benefit of the filing date of an earlier filed application under 35 U.S.C. §§ 120, 121 or 365(c), the 20-year term of that application will be based upon the filing date of the earliest U.S. application that the application makes reference to under 35 U.S.C. §§ 120, 121 or 365(c). (35 U.S.C. § 154(a)(2) does not take into account, for the determination of the patent term, any application on which priority is claimed under 35 U.S.C. §§ 119, 365(a) or 365(b).) For a c-i-p application, applicant should review whether any claim in the patent that will issue is supported by an earlier application and, if not, the applicant should consider canceling the reference to the earlier filed application. The term of a patent is not based on a claim-by-claim approach. See Notice of April 14, 1995, 60 Fed. Reg. 20,195, at 20,205.*

WARNING: *When the last day of pendency of a provisional application falls on a Saturday, Sunday, or Federal holiday within the District of Columbia, any nonprovisional application claiming benefit of the provisional application* ***must*** *be filed prior to the Saturday, Sunday, or Federal holiday within the District of Columbia. See 37 C.F.R. § 1.78(a)(3).*

☒ The new application being transmitted claims the benefit of prior U.S. application(s). Enclosed are ADDED PAGES FOR NEW APPLICATION TRANSMITTAL WHERE BENEFIT OF PRIOR U.S. APPLICATION(S) CLAIMED.

3. Papers Enclosed

A. Required for filing date under 37 C.F.R. § 1.53(b) (Regular) or 37 C.F.R. § 1.153 (Design) Application

23 Pages of specification (Includes cover page)

3 Pages of claims

14 Sheets of drawing

WARNING: ***DO NOT*** *submit original drawings. A high quality copy of the drawings should be supplied when filing a patent application. The drawings that are submitted to the Office must be on strong, white, smooth, and non-shiny paper and meet the standards according to § 1.84. If corrections to the drawings are necessary, they should be made to the original drawing and a high-quality copy of the corrected original drawing then submitted to the Office. Only one copy is required or desired. For comments on proposed then-new 37 C.F.R. § 1.84, see Notice of March 9, 1988 (1990 O.G. 57-62).*

NOTE: "Identifying indicia, if provided, should include the application number or the title of the invention, inventor's name, docket number (if any), and the name and telephone number of a person to call if the Office is unable to match the drawings to the proper application. This information should be placed on the back of each sheet of drawing a minimum distance of 1.5 cm. (5/8 inch) down from the top of the page . . ." 37 C.F.R. § 1.84(c)).

(complete the following, if applicable)

☐ The enclosed drawing(s) are photograph(s), and there is also attached a "PETITION TO ACCEPT PHOTOGRAPH(S) AS DRAWING(S)." 37 C.F.R. § 1.84(b).

☐ formal

☒ informal

B. Other Papers Enclosed

3 Pages of declaration and power of attorney (as-filed in 09/134,246; 8/14/98)

1 Pages of abstract

19 Other Copy of sequence listing and submission statement as-filed in 09/134,246; 8/14/98

4. Additional papers enclosed

☒ Amendment to claims

☒ Cancel in this applications claims 11 before calculating the filing fee. (At least one original independent claim must be retained for filing purposes.)

☐ Add the claims shown on the attached amendment. (Claims added have been numbered consecutively following the highest numbered original claims.)

☒ Preliminary Amendment

☒ Information Disclosure Statement (37 C.F.R. § 1.98)

☒ Form PTO–1449 (PTO/SB/08A and 08B)

☐ Citations

☐ Declaration of Biological Deposit

☒ Submission of "Sequence Listing," computer readable copy and/or amendment pertaining thereto for biotechnology invention containing nucleotide and/or amino acid sequence. AS-FILED in 09/134,246 (8/14/98)

☐ Authorization of Attorney(s) to Accept and Follow Instructions from Representative

☐ Special Comments

☐ Other

5. Declaration or oath (including power of attorney)

NOTE: A newly executed declaration is not required in a continuation or divisional application provided that the prior nonprovisional application contained a declaration as required, the application being filed is by all or fewer than all the inventors named in the prior application, there is no new matter in the application being filed, and a copy of the executed declaration filed in the prior application (showing the signature or an indication thereon that it was signed) is submitted. The copy must be accompanied by a statement requesting deletion of the names of person(s) who are not inventors of the application being filed. If the declaration in the prior application was filed under § 1.47, then a copy of that declaration must be filed accompanied by a copy of the decision granting § 1.47 status or, if a nonsigning person under § 1.47 has subsequently joined in a prior application, then a copy of the subsequently executed declaration must be filed. See 37 C.F.R. §§ 1.63(d)(1)-(3).

NOTE: A declaration filed to complete an application must be executed, identify the specification to which it is directed, identify each inventor by full name including family name and at least one given name, without abbreviation together with any other given name or initial, and the residence, post office address and country or citizenship of each inventor, and state whether the inventor is a sole or joint inventor. 37 C.F.R. § 1.63(a)(1)-(4).

NOTE: "The inventorship of a nonprovisional application is that inventorship set forth in the oath or declaration as prescribed by § 1.62, except as provided for in § 1.53(d)(4) and § 1.63(d). If an oath or declaration as prescribed by § 1.63 is not filed during the pendency of a nonprovisional application, the inventorship is that inventorship set forth in the application papers filed pursuant to § 1.53(b), unless a petition under this paragraph accompanied by the fee set forth in § 1.17(i) is filed supplying or changing the name or names of the inventor or inventors." 37 C.F.R. § 1.41(a)(1).

☒ Enclosed (copy of Declaration as-filed in 09/134,246; 8/14/98)

Executed by

(check **all** *applicable boxes)*

☒ inventor(s).

☐ legal representative of inventor(s). 37 C.F.R. §§ 1.42 or 1.43.

☐ joint inventor or person showing a proprietary interest on behalf of inventor who refused to sign or cannot be reached.

☐ This is the petition required by 37 C.F.R. § 1.47 and the statement required by 37 C.F.R. § 1.47 is also attached. See item 13 below for fee.

☐ Not Enclosed.

NOTE: Where the filing is a completion in the U.S. of an International Application or where the completion of the U.S. application contains subject matter in addition to the International Application, the application may be treated as a continuation or continuation-in-part, as the case may be, utilizing ADDED PAGE FOR NEW APPLICATION TRANSMITTAL WHERE BENEFIT OF PRIOR U.S. APPLICATION CLAIMED.

☐ Application is made by a person authorized under 37 C.F.R. § 1.41(c) on behalf of *all* the above named inventor(s).

(The declaration or oath, along with the surcharge required by 37 C.F.R. § 1.16(e) can be filed subsequently).

☐ Showing that the filing is authorized.
(not required unless called into question. 37 C.F.R. § 1.41(d))

6. Inventorship Statement

WARNING: *If the named inventors are each not the inventors of all the claims an explanation, including the ownership of the various claims at the time the last claimed invention was made, should be submitted.*

The inventorship for all the claims in this application are:

☒ The same.

or

☐ Not the same. An explanation, including the ownership of the various claims at the time the last claimed invention was made,

☐ is submitted.

☐ will be submitted.

7. Language

NOTE: An application including a signed oath or declaration may be filed in a language other than English. An English translation of the non-English language application and the processing fee of $130.00 required by 37 C.F.R. § 1.17(k) is required to be filed with the application, or within such time as may be set by the Office. 37 C.F.R. § 1.52(d).

☒ English

☐ Non-English

☐ The attached translation includes a statement that the translation is accurate. 37 C.F.R. § 1.52(d).

8. Assignment

☐ An assignment of the invention to _____

_____

☐ is attached. A separate ☐ "COVER SHEET FOR ASSIGNMENT (DOCUMENT) ACCOMPANYING NEW PATENT APPLICATION" or ☐ FORM PTO 1595 is also attached.

☐ will follow.

NOTE: "If an assignment is submitted with a new application, send two separate letters-one for the application and one for the assignment." Notice of May 4, 1990 (1114 O.G. 77-78).

WARNING: *A newly executed "CERTIFICATE UNDER 37 C.F.R. § 3.73(b)" must be filed when a continuation-in-part application is filed by an assignee. Notice of April 30, 1993, 1150 O.G. 62-64.*

9. Certified Copy

Certified copy(ies) of application(s)

Country	Appln. No.	Filed
Country	Appln. No.	Filed
Country	Appln. No.	Filed

from which priority is claimed

☐ is (are) attached.

☐ will follow.

NOTE: The foreign application forming the basis for the claim for priority must be referred to in the oath or declaration. 37 C.F.R. § 1.55(a) and 1.63.

NOTE: This item is for any foreign priority for which the application being filed directly relates. If any parent U.S. application or International Application from which this application claims benefit under 35 U.S.C. § 120 is itself entitled to priority from a prior foreign application, then complete item 18 on the ADDED PAGES FOR NEW APPLICATION TRANSMITTAL WHERE BENEFIT OF PRIOR U.S. APPLICATION(S) CLAIMED.

10. Fee Calculation (37 C.F.R. § 1.16)

A. ☒ Regular application

CLAIMS AS FILED				
Number filed	Number Extra		Rate	Basic Fee 37 C.F.R. § 1.16(a) $690.00
Total Claims (37 C.F.R. § 1.16(c)) 12	− 20 =	×	$ 18.00	0
Independent Claims (37 C.F.R. § 1.16(b)) 2	− 3 =	×	$ 78.00	0
Multiple dependent claim(s), if any (37 C.F.R. § 1.16(d))		+	$260.00	260.00

☐ **Amendment cancelling extra claims is enclosed.**

☐ **Amendment deleting multiple-dependencies is enclosed.**

☐ **Fee for extra claims is not being paid at this time.**

***NOTE:** If the fees for extra claims are not paid on filing they must be paid or the claims cancelled by amendment, prior to the expiration of the time period set for response by the Patent and Trademark Office in any notice of fee deficiency. 37 C.F.R. § 1.16(d).*

Filing Fee Calculation $ 950.00

B. ☐ Design application ($310.00—37 C.F.R. § 1.16(f))

Filing Fee Calculation $_____

C. ☐ Plant application
($480.00—37 C.F.R. § 1.16(g))

Filing fee calculation $_____

11. Small Entity Statement(s)

☒ Statement(s) that this is a filing by a small entity under 37 C.F.R. § 1.9 and 1.27 is (are) attached. (as-filed in 09/134,246 filed 8/14/1998)

WARNING: *"Status as a small entity must be specifically established in each application or patent in which the status is available and desired. Status as a small entity in one application or patent does not affect any other application or patent, including applications or patents which are directly or indirectly dependent upon the application or patent in which the status has been established. The refiling of an application under § 1.53 as a continuation, division, or continuation-in-part (including a continued prosecution application under § 1.53(d)), or the filing of a reissue application requires a new determination as to continued entitlement to small entity status for the continuing or reissue application. A nonprovisional application claiming benefit under 35 U.S.C. § 119(e), 120, 121, or 365(c) of a prior application, or a reissue application may rely on a statement filed in the prior application or in the patent if the nonprovisional application or the reissue application includes a reference to the statement in the prior application or in the patent or includes a copy of the statement in the prior application or in the patent and status as a small entity is still proper and desired. The payment of the small entity basic statutory filing fee will be treated as such a reference for purposes of this section." 37 C.F.R. § 1.28(a)(2).*

WARNING: *"Small entity status must not be established when the person or persons signing the . . . statement can **unequivocally** make the required self-certification." M.P.E.P., § 509.03, 6th ed., rev. 2, July 1996 (emphasis added).*

(complete the following, if applicable)

☒ Status as a small entity was claimed in prior application 09 / 134,246, filed on Aug. 14, 1998, from which benefit is being claimed for this application under:

35 U.S.C. § ☒ 119(e),
☒ 120,
☒ 121,
☐ 365(c),

and which status as a small entity is still proper and desired.

☒ A copy of the statement in the prior application is included.

Filing Fee Calculation (50% of **A**, **B** or **C** above)

$ 475.00

NOTE: Any excess of the full fee paid will be refunded if small entitiy status is established and a refund request are filed within 2 months of the date of timely payment of a full fee. The two-month period is not extendable under § 1.136. 37 C.F.R. § 1.28(a).

12. Request for International-Type Search (37 C.F.R. § 1.104(d))

(complete, if applicable)

☐ Please prepare an international-type search report for this application at the time when national examination on the merits takes place.

13. Fee Payment Being Made at This Time

☐ Not Enclosed

☐ No filing fee is to be paid at this time.
(This and the surcharge required by 37 C.F.R. § 1.16(e) can be paid subsequently.)

☒ Enclosed

☒ Filing fee	$ 475.00
☐ Recording assignment ($40.00; 37 C.F.R. § 1.21(h)) (See attached "COVER SHEET FOR ASSIGNMENT ACCOMPANYING NEW APPLICATION".)	$ _____
☐ Petition fee for filing by other than all the inventors or person on behalf of the inventor where inventor refused to sign or cannot be reached ($130.00; 37 C.F.R. §§ 1.47 and 1.17(i))	$ _____
☐ For processing an application with a specification in a non-English language ($130.00; 37 C.F.R. §§ 1.52(d) and 1.17(k))	$ _____
☐ Processing and retention fee ($130.00; 37 C.F.R. §§ 1.53(d) and 1.21(l))	$ _____
☐ Fee for international-type search report ($40.00; 37 C.F.R. § 1.21(e))	$ _____

NOTE: 37 C.F.R. § 1.21(l) establishes a fee for processing and retaining any application that is abandoned for failing to complete the application pursuant to 37 C.F.R. § 1.53(f) and this, as well as the changes to 37 C.F.R. §§ 1.53 and 1.78(a)(1), indicate that in order to obtain the benefit of a prior U.S. application, either the basic filing fee must be paid, or the processing and retention fee of § 1.21(l) must be paid, within 1 year from notification under § 53(f).

Total fees enclosed $ 475.00

14. Method of Payment of Fees

☒ Check in the amount of $ 475.00

☐ Charge Account No. _____ in the amount of $_____.

A duplicate of this transmittal is attached.

NOTE: Fees should be itemized in such a manner that it is clear for which purpose the fees are paid. 37 C.F.R. § 1.22(b).

15. Authorization to Charge Additional Fees

WARNING: *If no fees are to be paid on filing, the following items should not be completed.*

WARNING: *Accurately count claims, especially multiple dependent claims, to avoid unexpected high charges, if extra claim charges are authorized.*

☒ The Commissioner is hereby authorized to charge the following additional fees by this paper and during the entire pendency of this application to Account No. 14-0740 :

- ☒ 37 C.F.R. § 1.16(a), (f) or (g) (filing fees)
- ☒ 37 C.F.R. § 1.16(b), (c) and (d) (presentation of extra claims)

NOTE: *Because additional fees for excess or multiple dependent claims not paid on filing or on later presentation must only be paid or these claims cancelled by amendment prior to the expiration of the time period set for response by the PTO in any notice of fee deficiency (37 C.F.R. § 1.16(d)), it might be best not to authorize the PTO to charge additional claim fees, except possibly when dealing with amendments after final action.*

- ☒ 37 C.F.R. § 1.16(e) (surcharge for filing the basic filing fee and/or declaration on a date later than the filing date of the application)
- ☒ 37 C.F.R. § 1.17(a)(1)–(5) (extension fees pursuant to § 1.136(a)).
- ☒ 37 C.F.R. § 1.17 (application processing fees)

NOTE: *". . .A written request may be submitted in an application that is an authorization to treat any concurrent or future reply, requiring a petition for an extension of time under this paragraph for its timely submission, as incorporating a petition for extension of time for the appropriate length of time. An authorization to charge all required fees, fees under § 1.17, or all required extension of time fees will be treated as a constructive petition for an extension of time in any concurrent or future reply requiring a petition for an extension of time under this paragraph for its timely submission. Submission of the fee set forth in § 1.17(a) will also be treated as a constructive petition for an extension of time in any concurrent reply requiring a petition for an extension of time under this paragraph for its timely submission." 37 C.F.R. § 1.136(a)(3).*

- ☐ 37 C.F.R. § 1.18 (issue fee at or before mailing of Notice of Allowance, pursuant to 37 C.F.R. § 1.311(b))

NOTE: *Where an authorization to charge the issue fee to a deposit account has been filed before the mailing of a Notice of Allowance, the issue fee will be automatically charged to the deposit account at the time of mailing the notice of allowance. 37 C.F.R. § 1.311(b).*

NOTE: *37 C.F.R. § 1.28(b) requires "Notification of any change in status resulting in loss of entitlement to small entity status must be filed in the application . . . prior to paying, or at the time of paying, . . . the issue fee. . . " From the wording of 37 C.F.R. § 1.28(b), (a) notification of change of status must be made even if the fee is paid as "other than a small entity" and (b) no notification is required if the change is to another small entity.*

16. Instructions as to Overpayment

NOTE: ". . . Amounts of twenty-five dollars or less will not be returned unless specifically requested within a reasonable time, nor will the payer be notified of such amounts; amounts over twenty-five dollars may be returned by check or, if requested, by credit to a deposit account." 37 C.F.R. § 1.26(a).

☒ Credit Account No. 14-0740

☐ Refund

SIGNATURE OF PRACTITIONER

Reg. No. 30901

Gregory D. Williams
General Counsel
(type or print name of attorney)

Tel. No. (978) 927-5054 X:292

New England Biolabs, Inc.
32 Tozer Road
P.O. Address
Beverly, MA 01915

Customer No.

☒ **Incorporation by reference of added pages**

(check the following item if the application in this transmittal claims the benefit of prior U.S. application(s) (including an international application entering the U.S. stage as a continuation, divisional or C-I-P application) and complete and attach the ADDED PAGES FOR NEW APPLICATION TRANSMITTAL WHERE BENEFIT OF PRIOR U.S. APPLICATION(S) CLAIMED)

☒ Plus Added Pages for New Application Transmittal Where Benefit of Prior U.S. Application(s) Claimed

Number of pages added 5

☒ Plus Added Pages for Papers Referred to in Item 4 Above

Number of pages added 25

☐ Plus added pages deleting names of inventor(s) named in prior application(s) who is/are no longer inventor(s) of the subject matter claimed in this application.

Number of pages added _____

☐ Plus "Assignment Cover Letter Accompanying New Application"

Number of pages added _____

☐ **Statement Where No Further Pages Added**

(if no further pages form a part of this Transmittal, then end this Transmittal with this page and check the following item)

☐ This transmittal ends with this page.

Practitioner's Docket No. NEB-135-C **PATENT**

ADDED PAGES FOR APPLICATION TRANSMITTAL WHERE BENEFIT OF PRIOR U.S. APPLICATION(S) CLAIMED

NOTE: See 37 C.F.R. § 1.78.

17. Relate Back

WARNING: *If an application claims the benefit of the filing date of an earlier filed application under 35 U.S.C. §§ 120, 121 or 365(c), the 20-year term of that application will be based upon the filing date of the earliest U.S. application that the application makes reference to under 35 U.S.C. §§ 120, 121 or 365(c). (35 U.S.C. § 154(a)(2) does not take into account, for the determination of the patent term, any application on which priority is claimed under 35 U.S.C. §§ 119, 365(a) or 365(b).) For a c-i-p application, applicant should review whether any claim in the patent that will issue is supported by an earlier application and, if not, the applicant should consider canceling the reference to the earlier filed application. The term of a patent is not based on a claim-by-claim approach. See Notice of April 14, 1995, 60 Fed. Reg. 20,195, at 20,205.*

(complete the following, if applicable)

☒ Amend the specification by inserting, before the first line, the following sentence:

A. 35 U.S.C. § 119(e)

NOTE: "Any nonprovisional application claiming the benefit of one or more prior filed copending provisional applications must contain or be amended to contain in the first sentence of the specification following the title a reference to each such prior provisional application, identifying it as a provisional application, and including the provisional application number (consisting of series code and serial number)." 37 C.F.R. § 1.78(a)(4).

☐ "This application claims the benefit of U.S. Provisional Application(s) No(s).:

APPLICATION NO(S).:	FILING DATE
09 / 134,246	Aug. 14, 1998 "
/	"
/	"

B. 35 U.S.C. §§ 120, 121 and 365(c)

NOTE: "Except for a continued prosecution application filed under § 1.53(d), any nonprovisional application claiming the benefit of one or more prior filed copending nonprovisional applications or international applications designating the United States of America must contain or be amended to contain in the first sentence of the specification following the title a reference to each such prior application, identifying it by application number (consisting of the series code and serial number) or international application number and international filing date and indicating the relationship of the applications. . . . Cross-references to other related applications may be made when appropriate." (See § 1.14(a)). 37 C.F.R. § 1.78(a)(2).

☒ "This application is a

- ☒ continuation
- ☐ continuation-in-part
- ☐ divisional

of copending application(s)

☒ application number 09 / 134,246 filed on 8/14/98 "

☐ International Application _____ filed on _____ and which designated the U.S."

NOTE: The proper reference to a prior filed PCT application that entered the U.S. national phase is the U.S. serial number and the filing date of the PCT application that designated the U.S.

NOTE: (1) Where the application being transmitted adds subject matter to the International Application, then the filing can be as a continuation-in-part or (2) if it is desired to do so for other reasons then the filing can be as a continuation.

NOTE: The deadline for entering the national phase in the U.S. for an international application was clarified in the Notice of April 28, 1987 (1079 O.G. 32 to 46) as follows:

"The Patent and Trademark Office considers the International application to be pending until the 22nd month from the priority date if the United States has been designated and no Demand for International Preliminary Examination has been filed prior to the expiration of the 19th month from the priority date and until the 32nd month from the priority date if a Demand for International Preliminary Examination which elected the United States of America has been filed prior to the expiration of the 19th month from the priority date, provided that a copy of the international application has been communicated to the Patent and Trademark Office within the 20 or 30 month period respectively. If a copy of the international application has not been communicated to the Patent and Trademark Office within the 20 or 30 month period respectively, the international application becomes abandoned as to the United States 20 or 30 months from the priority date respectivley. These periods have been placed in the rules as paragraph (h) of § 1.494 and paragraph (i) of § 1.495. A continuing application under 35 U.S.C. 365(c) and 120 may be filed anytime during the pendency of the international application."

☐ "The nonprovisional application designated above, namely application _____ / _____, filed _____, claims the benefit of U.S. Provisional Application(s) No(s).:

APPLICATION NO(S).:	**FILING DATE**
_____ / _____	_____ "
_____ / _____	_____ "
_____ / _____	_____ "

☐ Where more than one reference is made above, please combine all references into one sentence.

18. Relate Back—35 U.S.C. § 119 Priority Claim for Prior Application

The prior U.S. application(s), including any prior International Application designating the U.S., identified above in item 17B, in turn itself claim(s) foreign priority(ies) as follows:

Country	Appln. no.	Filed on

The certified copy(ies) has (have)

☐ been filed on _____, in prior application 0 /_____, which was filed on _____.

☐ is (are) attached.

WARNING: *The certified copy of the priority application that may have been communicated to the PTO by the International Bureau may **not** be relied on without any need to file a certified copy of the priority application **in the continuing application.** This is so because the certified copy of the priority application communicated by the International Bureau is placed in a folder and is not assigned a U.S. serial number unless the national stage is entered. Such folders are disposed of if the national stage is not entered. Therefore, such certified copies may not be available if needed later in the prosecution of a continuing application. An alternative would be to physically remove the priority documents from the folders and transfer them to the continuing application. The resources required to request transfer, retrieve the folders, make suitable record notations, transfer the certified copies, enter and make a record of such copies in the Continuing Application are substantial. Accordingly, the priority documents in folders of international applications that have not entered the national stage may not be relied on. Notice of April 28, 1987 (1079 O.G. 32 to 46).*

19. Maintenance of Copendency of Prior Application

NOTE: The PTO finds it useful if a copy of the petition filed in the prior application extending the term for response is filed with the papers constituting the filing of the continuation application. Notice of November 5, 1985 (1060 O.G. 27).

A. ☒ Extension of time in prior application

*(This item **must** be completed and the papers filed **in the prior application,** if the period set in the prior application has run.)*

☒ A petition, fee and response extends the term in the pending **prior** application until 10/6/00.

☒ A **copy** of the petition filed in prior application is attached.

B. ☐ Conditional Petition for Extension of Time in Prior Application

(complete this item, if previous item not applicable)

☐ A conditional petition for extension of time is being filed in the pending **prior** application.

☐ A **copy** of the conditional petition filed in the prior application is attached.

20. Further Inventorship Statement Where Benefit of Prior Application(s) Claimed

(complete applicable item (a), (b) and/or (c) below)

(a) ☒ This application discloses and claims only subject matter disclosed in the prior application whose particulars are set out above and the inventor(s) in this application are

- ☒ the same.
- ☐ less than those named in the prior application. It is requested that the following inventor(s) identified for the prior application be deleted:

_____

(type name(s) of inventor(s) to be deleted)

(b) ☐ This application discloses and claims additional disclosure by amendment and a new declaration or oath is being filed. With respect to the prior application, the inventor(s) in this application are

- ☐ the same.
- ☐ the following additional inventor(s) have been added:

_____

(type name(s) of inventor(s) to be added)

(c) The inventorship for all the claims in this application are

- ☒ the same.
- ☐ not the same. An explanation, including the ownership of the various claims at the time the last claimed invention was made
  - ☐ is submitted.
  - ☐ will be submitted.

21. Abandonment of Prior Application *(if applicable)*

☐ Please abandon the prior application at a time while the prior application is pending, or when the petition for extension of time or to revive in that application is granted, and when this application is granted a filing date, so as to make this application copending with said prior application.

NOTE: According to the Notice of May 13, 1983 (103, TMOG 6-7), the filing of a continuation or continuation-in-part application is a proper response with respect to a petition for extension of time or a petition to revive and should include the express abandonment of the prior application conditioned upon the granting of the petition and the granting of a filing date to the continuing application.

22. Petition for Suspension of Prosecution for the Time Necessary to File an Amendment

WARNING: *"The claims of a new application may be finally rejected in the first Office action in those situations where (A) the new application is a continuing application of, or a substitute for, an earlier application, and (B) all the claims of the new application (1) are drawn to the same invention claimed in the earlier application, and (2) would have been properly finally rejected on the grounds of art of record in the next Office action if they had been entered in the earlier application." M.P.E.P., § 706.07(b), 7th ed.*

NOTE: Where it is possible that the claims on file will give rise to a first action final for this continuation application and for some reason an amendment cannot be filed promptly (e.g., experimental data is being gathered) it may be desirable to file a petition for suspension of prosecution for the time necessary.

(check the next item, if applicable)

☐ There is provided herewith a Petition To Suspend Prosecution for the Time Necessary to File An Amendment (New Application Filed Concurrently)

23. Small Entity (37 C.F.R. § 1.28(a))

☒ Applicant has established small entity status by the filing of a statement in parent application09 /134,246 on 8/14/98 .

☒ A copy of the statement previously filed is included.

WARNING: *See 37 C.F.R. § 1.28(a).*

WARNING: *"Small entity status must not be established when the person or persons signing the . . . statement can **unequivocally** make the required self-certification." M.P.E.P., § 509.03, 7th ed. (emphasis added).*

24. NOTIFICATION IN PARENT APPLICATION OF THIS FILING

☒ A notification of the filing of this
(check one of the following)

- ☒ continuation
- ☐ continuation-in-part
- ☐ divisional

is being filed in the parent application, from which this application claims priority under 35 U.S.C. § 120.

COPY

Practitioner's Docket No. NEB-135 **PATENT**

☒ Applicant Wayne, et al. ☐ Patentee _____

☐ Application No. ☐ Patent No. _____

☐ Filed on ☐ Issued on _____

Title: Method For Construction Of Thermus-E. coli Shuttle Vectors And Identification Of Two Thermus Plasmid Replication Origins

STATEMENT CLAIMING SMALL ENTITY STATUS (37 CFR 1.9(f) and 1.27(c))—SMALL BUSINESS CONCERN

I hereby state that I am

☐ the owner of the small business concern identified below:

☒ an official of the small business concern empowered to act on behalf of the concern identified below:

Name of Small Business Concern New England Biolabs, Inc.

Address of Small Business Concern 32 Tozer Road

Beverly, MA 01915

I hereby state that the above identified small business concern qualifies as a small business concern, as defined in 13 CFR 121.12, and reproduced in 37 CFR 1.9(d), for purposes of paying reduced fees to the United States Patent and Trademark Office under Sections 41(a) and (b) of Title 35, United States Code, in that the number of employees of the concern, including those of its affiliates, does not exceed 500 persons. For purposes of this statement, (1) the number of employees of the business concern is the average over the previous fiscal year of the concern of the persons employed on a full-time, part-time or temporary basis during each of the pay periods of the fiscal year, and (2) concerns are affiliates of each other when either, directly or indirectly, one concern controls or has the power to control the other, or a third-party or parties controls or has the power to control both.

I hereby state that rights under contract or law have been conveyed to, and remain with, the small business concern identified above, with regard to the invention described in

☐ the specification filed herewith, with title as listed above.

☒ the application identified above.

☐ the patent identified above.

If the rights held by the above-identified small business concern are not exclusive, each individual, concern or organization having rights in the invention is listed below* and no rights to the invention are held by any person, other than the inventor, who would not qualify as an independent inventor under 37 CFR 1.9(c), if that person made the invention, or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e).

*NOTE: *Separate statements are required from each named person, concern or organization having rights to the invention as to their status as small entities. (37 CFR 1.27)*

Each such person, concern or organization having any rights in the invention is listed below:

☐ No such person, concern, or organization exists.

☒ Each such person, concern or organization is listed below.

Name New England Biolabs, Inc.

Address 32 Tozer Road; Beverly, MA 01915

☐ INDIVIDUAL ☒ SMALL BUSINESS CONCERN ☐ NONPROFIT ORGANIZATION

Name _____

Address _____

☐ INDIVIDUAL ☐ SMALL BUSINESS CONCERN ☐ NONPROFIT ORGANIZATION

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small business entity is no longer appropriate. (37 CFR 1.28(b))

(check the following item, if desired)

NOTE: The following verification statement need not be made in accordance with the rules published on Oct. 10, 1997, 62 Fed. Reg. 52,131, effective Dec. 1, 1997.

NOTE: "The presentation to the Office (whether by signing, filing, submitting, or later advocating) of any paper by a party, whether a practitioner or non-practitioner, constitutes a certification under § 10.18(b) of this chapter. Violations of § 10.18(b)(2) of this chapter by a party, whether a practitioner or non-practitioner, may result in the imposition of sanctions under § 10.18(c) of this chapter. Any practitioner violating § 10.18(b) may also be subject to disciplinary action. See §§ 10.18(d) and 10.23(c)(15)." 37 C.F.R. § 1.4(d)(2).

☒ I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

Name of Person Signing Gregory D. Williams

Title of Person if Other Than Owner General Counsel

Address of Person Signing New England Biolabs, Inc
32 Tozer Road; Beverly, MA 01915

SIGNATURE _____ Date 8/13/98

Docket No.: NEB-135-C

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Wayne, et al. Examiner:

Application No.: Group No.:

Filing Date:

Title: Method For Construction of *Thermus-E. coli* Shuttle Vectors and Identification of Two *Thermus* Plasmid Replication Origins

Honorable Commissioner of Patents
and Trademarks
Washington, D.C. 20231

Sir:

PRELIMINARY AMENDMENT
INFORMATION DISCLOSURE STATEMENT
AND
SEQUENCE LISTING

This is a Continuation Application of U.S. Application No. 09/134,246 filed August 14, 1998. Preliminary to examination on the merits, please amend the Application as follows:

IN THE SPECIFICATION

--RELATED APPLICATIONS

This is a Continuation Application of U.S. Application No. 09/134,246 filed August 14, 1998.--

IN THE CLAIMS

Please amend claim 2 as follows:

2. (amended) A recombinant plasmid comprising at least one *Thermus sp.* replication origin, wherein said replication origin [includes] is contained within the isolated DNA sequence of claim 1.

Please amend claim 3 as follows:

3. (amended) The recombinant plasmid of claim 2, further comprising at least one promoter sequence selected from the [group consisting of the] DNA sequence of [SEQ ID NO:6] SEQ ID NO:5, -10 TATTTT, -35, TTGCCA, 17 bp spacing; or -10 TAGGGT, -35, TTGCCC, 18 bp spacing [residues 27-32 of SEQ ID NO:6, residues 50-55 of SEQ ID NO:6, residues 86-90 of SEQ ID NO:6, and residues 109-114 of SEQ ID NO:6].

Please amend claim 6 as follows:

6. (amended) An isolated DNA encoding a *Thermus sp.* [Promoter] promoter, wherein said isolated DNA is selected from the [group consisting of the] DNA sequence of [SEQ ID NO:6] SEQ ID NO:5, -10 TATTTT, -35, TTGCCA, 17 bp spacing; or -10 TAGGGT, -35, TTGCCC, 18 bp spacing [residues 27-32 of SEQ ID NO:6, residues 50-55 of SEQ ID NO:6, residues 86-90 of SEQ ID NO:6, and residues 109-114 of SEQ ID NO:6].

Please cancel claim 11 without prejudice.

REMARKS

Claims 2, 3 and 6 have been amended to more particularly define the present invention. Claim 11 has been cancelled without prejudice as

this claim is pending in U.S. Application Serial No. 09/134,246. No new matter has been added by virtue of these additional claims.

Applicants have filed this Continuation Application to claim subject matter disclosed in the Application as originally filed. This amendment does not add any new matter and the claims presented are believed patentable.

INFORMATION DISCLOSURE STATEMENT

In accordance with the provisions of 37 C.F.R. §1.56, §1.97 and §1.98, Applicants wish to bring the following references, References AA-AQ cited on the attached PTO-1449 Form to the attention of the Examiner.

Copies of References AA-AQ were previously submitted by Applicant in corresponding U.S. Application Serial No. 09/134,246 filed August 14, 1998.

Applicants respectfully submit that since the present Application claims priority under 09/134,246 filed on August 14, 1998 which claims priority under 35 U.S.C. §120 in accordance with 37 CFR §1.97(d), a copy of the above-identified References need not be provided. However, in the event that the Examiner requires an additional copy of any of the cited References AA-AQ, the Examiner is requested to contact the undersigned who will provide the requested copies.

It is respectfully submitted that each of the documents shown on PTO-1449 be made of record in this Application.

SEQUENCE LISTING

Applicants respectfully request that the Sequence Listing submitted on August 14, 1998 in corresponding U.S. Application Serial No. 09/134,246 filed August 14, 1998 be transferred to this Application, in accordance with 37 C.F.R. §1.821(e), the computer readable copy from Applicant's other Application identified as follows:

In re Application of: Wayne, et al.

Application No.: 09/134,246 Group No.: 1636

Filed: August 14, 1998 Examiner: W. Sandals

For: Method For Construction of Thermus-E. coli Shuttle Vectors And Identification of Two Thermus Plasmid Replication Origins

The sequence identifiers of Applicants other Application directly corresponds to the sequence identifiers of the instant Application.

Early and favorable consideration leading to prompt issuance of this Application is earnestly solicited.

Should the Examiner wish to discuss any of the amendments and/or remarks made herein, the undersigned attorney would appreciate the opportunity to do so. Thus, the Examiner is hereby authorized to call the undersigned collect at the number shown below.

Respectfully submitted,

NEW ENGLAND BIOLABS, INC.

Date: 9/13/00

Gregory D. Williams
(Reg. No.: 30901)
Attorney for Applicant
32 Tozer Road
Beverly, Massachusetts 01915
(978) 927-5054; ext. 292

Docket No. NEB-135

COPY

IN THE UNITED STATES PATENT OFFICE AND TRADEMARK OFFICE
APPLICATION FOR UNITED STATES LETTERS PATENT

INVENTOR(S): Jay Wayne
Shuang-yong Xu

TITLE: METHOD FOR CONSTRUCTION OF *THERMUS-E. COLI* SHUTTLE VECTORS AND IDENTIFICATION OF TWO *THERMUS* PLASMID REPLICATION ORIGINS

ATTORNEY: Gregory D. Williams
General Counsel
NEW ENGLAND BIOLABS, INC.
32 Tozer Road
Beverly, Massachusetts 01915
(978) 927-5054; Ext. 292

EXPRESS MAILING LABEL NO.: IB442856348US

METHOD FOR CONSTRUCTION OF *THERMUS-E. COLI* SHUTTLE VECTORS AND IDENTIFICATION OF TWO *THERMUS* PLASMID REPLICATION ORIGINS

BACKGROUND OF THE INVENTION

The present invention relates to recombinant DNA molecules encoding plasmid DNA replication origins in *Thermus*, as well as to shuttle vectors which contain the same.

Many species of bacteria contain small circular extrachromosomal genetic elements, known as plasmids. Plasmids have been found in a number of bacteria which live in extreme environments, including the thermophiles, which live at high temperatures of more than 55°C (Munster et al., *Appl. Environ. Microbiol.* 50:1325-1327 (1985); Kristjansson and Stetter, in 'Thermophilic Bacteria', Kristjansson, ed., p. 1-18 (1992)). However, most thermophile plasmids remain 'cryptic' in that functional genes have not been isolated from them, hence leaving their functional significance speculative (Hishinuma et al., *J. Gen. Microbiol.* 104:193-199 (1978); Eberhard et al., *Plasmid* 6:1-6 (1981); Vásquez et al., *FEBS Lett.* 158:339-342 (1983)). Common genes found in plasmids include those encoding plasmid replication and cellular maintenance, antibiotic resistance, bacteriocin production, sex determination, and other cellular functions (Kornberg and Baker, 'DNA Replication', 2nd ed. (1991)).

It is often particularly difficult to cultivate thermophilic bacteria within the laboratory. They require high temperatures and often-unknown environmental conditions for acceptable growth (Kristjansson and Stetter, in 'Thermophilic Bacteria', Kristjansson, ed., p. 1-18 (1992)). However, with the advent of genetic engineering, it is now possible to clone genes from thermophiles into more easily cultivatable laboratory organisms, such as *E. coli* (Kristjansson, *Trends Biotech.* 7:349-353 (1989); Coolbear et al., *Adv. Biochem. Eng. Biotech.* 45:57-98 (1992)). The expression of such genes can be finely controlled within *E. coli.*

A *Thermus-E. coli* shuttle vector would be desirable if one needs to have the convenience of cloning in *E. coli*, isolation of DNA from *E. coli* for further manipulations and subsequently gene selection and expression in *Thermus.* Such *Thermus-E. coli* shuttle vectors could be used to screen, select and express thermostable proteins in *Thermus.* Using these vectors, a gene could, for example, be mutated within a mesophile, transferred to a thermophile, and then its encoded protein selected for increased thermostability. In this way, mesophile-thermophile shuttle-vectors can be used to conduct directed evolution, or protein engineering, on desirable gene products.

There is commercial incentive to produce thermostable proteins which are usually more thermostable in denaturing conditions then mesophilic counterparts (Wiegel and

Ljungdahl, *CRC Crit. Rev. Biotech.* 3:39-108 (1984); Kristjansson, *Trends Biotech.* 7:349-353 (1989); Coolbear et al., *Adv. Biochem. Eng. Biotech.* 45:57-98 (1992)). These thermostable enzymes can also be used in a variety of assays, such as PCR, restriction enzyme-mediated PCR, thermo-cycle DNA sequencing and strand-displacement amplification, in which high temperatures are desirable. The shuttle vectors of the present invention should facilitate production of such thermostable proteins.

SUMMARY OF THE INVENTION

The present invention relates to recombinant DNA molecules encoding plasmid DNA replication origins in *Thermus*, as well as to shuttle vectors which contain the same.

Mesophile-thermophile shuttle vectors require origins of replication (*oris*) to be genetically maintained and transferred within each bacterial species. To construct appropriate mesophile-thermophile shuttle-vectors, restriction digested thermophile plasmid DNA fragments were ligated into the mesophilic vector pUC19-Km^R (the thermostable Km^R marker can be selected at 50°-65°C). Plasmid pUC19 uses the ColEI *ori* to replicate within *E. coli*, and does not replicate within the plasmid-accepting thermophile *Thermus thermophilus* HB27 or HB27 Pro^- (Koyama et al., *J. Bacteriol.* 166:338-340 (1986)). We reasoned that the introduction of plasmid DNA

from related *Thermus* species, which contained a complete thermophilic *ori*, would confer plasmid replication within HB27.

The thermophilic eubacterium *Thermus* species YS45 (Raven et al., *Nucl. Acids Res.* 21:4397 (1993)) contains two cryptic plasmids, and grows between 55°C and 70°C. These two *Thermus* plasmids were named pTsp45S and pTsp45L. These plasmids were digested with a variety of restriction endonucleases to produce fragments that can be cloned into pUC19-derived vectors. A pUC19-derived plasmid with a 4.2-kb *Xba*I fragment of the small plasmid (pTsp45S, 5.8 kb) of YS45 replicated within HB27. Therefore this *Xba*I fragment must contain a thermophilic *ori*. Subsequent deletion analysis revealed that only 2.3 kb (an *Nhe*I fragment) within the 4.2 kb was necessary for thermophilic plasmid replication, and that it encodes a replication protein (RepT). The *repT* gene encodes the 341 amino acid protein, RepT, with predicted molecular mass of 38.2 kDa.

A second *Thermus* plasmid replication origin from pTsp45L was defined within a 9 kb *Sph*I fragment. This fragment encodes a gene (*parA*) for plasmid replication and partition. It also contains direct repeats of 5' RRCTTTTYYY 3' (SEQ ID NO:1), 5' RRYTTTG 3' (SERQ ID NO:2), and an inverted repeat of

5' TTAACCTTTTTTCAAGAAAAAGAGATAA 3' (SEQ ID NO:3)
3' AATTGGAAAAAAGTT CTTTTTCTCTATT 5'
(COMPLEMENT OF SEQ ID NO:3)

The direct repeats and inverted repeats are important for pTsp45L plasmid replication. Deletion of these repeats abolished replication activity in *Thermus*.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is the DNA sequence (SEQ ID NO:4) of *repT* gene from pTsp45S and its encoded amino acid sequence.

Figure 2 is the promotor sequence (SEQ ID NO:5) upstream of *repT* gene.

Figure 3 is the entire DNA sequence (SEQ ID NO:6) of *Thermus* plasmid pTsp45S.

Figure 4 illustrates the genetic organization of *Thermus* plasmid pTsp45S. The gene *repT* encodes RepT for plasmid replication.

Figure 5 is the *parA* DNA sequence from pTsp45L and the encoded amino acid sequence (SEQ ID NO:7).

Figure 6 is the seven open reading frames encoded by pTsp45L. Frames a, b, and c are encoded by the top strand. Frames d, e, and f are encoded by the bottom strand.

Figure 7 is the entire DNA sequence of *Thermus* plasmid pTsp45L (SEQ ID NO:8).

DETAILED DESCRIPTION OF THE INVENTION

The method described herein by which a *Thermus* plasmid replication origin is preferably cloned and selected comprises the following steps:

1. The plasmid DNA of a target host, such as *Thermus* species YS45 plasmid pTsp45S and pTsp45L, is purified.

2. The plasmid DNA is digested with appropriate restriction endonucleases; for *Thermus* species YS45, *Hin*dIII, *Kpn*I, *Pst*I, *Sph*I, and *Xba*I are used to generate 1 to 12 kb restriction fragments. This map is used to orient and localize genes within the plasmid.

3. The digested plasmid DNA is then ligated into similarly cleaved/CIP treated vectors such as pUC-EKR or pUC-EKF (Ap^R at 37°C, Km^R at 50-65°C) cloning vectors. The ligated DNA is used to transform an appropriate host, e.g., a $HsdR^-$, $McrBC^-$, Mrr^- strain, such as *E. coli* strain RR1. The DNA/cell mixtures are then plated on ampicillin selective media to grow only transformed cells to form primary restriction libraries, such as *Hin*dIII, *Kpn*I, *Pst*I, *Sph*I, and *Xba*I DNA libraries for Thermus species YS45.

4. The recombinant plasmids are purified to form the primary plasmid library that might contain thermophilic

plasmid origins. Plasmids are digested *in vitro* with a variety of endonucleases to confirm DNA inserts.

5. The plasmid DNA libraries are used to transform an appropriate thermophilic host cell such as *Thermus thermophilus* HB27 (Pro^-) cells and transformants are selected on Km plates at 60°-65°C for 48 hours.

6. Individual Km^R transformants are amplified in small culture at 65°C and plasmid DNA is isolated from the overnight cell culture. The plasmid DNA is then digested with an appropriate restriction endonuclease (e.g., *Hin*dIII, *Kpn*I, *Pst*I, *Sph*I, or *Xba*I) to cut out the *Thermus* DNA insert.

7. One clone from the *Xba*I library described above contained a 4.2 kb *Thermus* DNA which replicates in both *Thermus* and *E. coli*. The 4.2 kb insert DNA of the recombinant pUC-EKF clone was sequenced. To facilitate sequencing, the insert DNA wass further sub-cloned within pUC19 based upon preliminary sequence and mapping. The sequenced DNA was then assembled to match that of the thermophilic plasmid map. The remaining DNA fragments from pTsp45S were also cloned and sequenced. In this way, the thermophilic plasmid (pTsp45S) was completely sequenced.

8. To reduce the size of the *Thermus* replication origin, the 4.2 kb *Xba*I fragment was further digested with restriction enzymes and subcloned into pUC-EKF or pUC-EKR.

One recombinant plasmid contained a 2.3 kb *Nhe*I fragment that replicates in *Thermus* and *E. coli*. This plasmid pUC-EKF-Tsp3 is a *Thermus-E. coli* shuttle vector.

9. One open reading frame of 1026 bp encoding a 341-amino acid protein was found within the *Thermus* origin. Deletion of 234 bp (78 amino acid residues) within this gene abolished the *Thermus* replication function. Insertion of stop codons within this gene causes premature termination and negates the *Thermus* transformation. Therefore it was determined that this gene (*repT*) is required for plasmid replication in *Thermus* HB27 (Pro^-) cells.

10. Two *Thermus* promoters were found upstream of the *repT* gene that are important for *repT* expression.

11. Plasmid pTsp45L (a mixture of pTsp45L and pTsp45S) was digested with *Hin*dIII, *Kpn*I, *Pst*I, *Sph*I, or *Xba*I. The digested DNA fragments were cloned into pUC-EKR vector to produce *Thermus* DNA libraries for subsequent selection of *Thermus* plasmid replication origin(s).

12. Approximately 450 Ap^R transformants were derived from pUC-EKR + *Hin*dIII fragments, + *Kpn*I fragments, + *Pst*I fragments, + *Sph*I fragments, and + *Xba*I fragments, respectively. pUC-EKR plasmids with *Hin*dIII, *Kpn*I, *Pst*I, *Sph*I, or *Xba*I fragment inserts were amplified in *E.coli*.

13. The DNA libraries were used to transform *Thermus thermophilus* HB27 (Pro$^-$). Transformants were plated on Km plates and incubated at 60°C for two days. Plasmid DNA was extracted from seventeen Km^R transformants and digested with *Xba*I, *Pst*I, or *Sph*I. Restriction mapping and Southern blot analysis were carried out.

14. The 9 kb *Sph*I *Thermus* origin insert and the 12 kb *Thermus* origin insert were from pTsp45L. The entire pTsp45L plasmid can be separated into two *Sph*I fragments, 3 kb and 9 kb respectively. The 9 kb *Sph*I fragment contains the functional *Thermus* replication origin. The inserts were sequenced by using pUC19 universal forward and reverse primers and by primer walking. Plasmid pTsp45L is 11958 bp, encoding 7 possible genes.

15. Orf3 is most likely the candidate for pTsp45L replication protein, because it has homolgy to RepA protein of *Agrobacterium* plasmid pTiB6S3, replication protein of *Agrobacterium* plasmid pRiA4b, plasmid partition protein of *Borrelia*, partition protein of *Frankia*, RepA protein of *Rhizobium*, and DNA partition protein ParA of *Caulobacter*. Orf2 may be an accessary protein for pTsp45L plasmid replication. Orf3 was renamed as *parA* gene.

16. There are direct repeats and inverted repeats in the 9 kb *Sph*I fragment containing the functional replication origin. The direct repeats I are:

5' GGCTTTTCTT 3' (SEQ ID NO:9)
5' AACTTTTCCC 3' (SEQ ID NO:10)
5' GACTTTTTTC 3' (SEQ ID NO:11)
consensus 5' RRCTTTTYYY 3' (SEQ ID NO:1)

The direct repeats II are:

5' AACTTTG 3' (SEQ ID NO:12)
5' AGTTTTG 3' (SEQ ID NO:13)
5' GATTTTG 3' (SEQ ID NO:14)
5' AACTTTG 3' (SEQ ID NO:15)
consensus 5' RRYTTTG 3' (SEQ ID NO:2)

The inverted repeat is:

5' TTAACCTTTTTTCAAGAAAAAGAGATAA 3' (SEQ ID NO:3)
3' AATTGGAAAAAAGTT CTTTTTCTCTATT 5'
(COMPLEMENT OF SEQ ID NO:3)

(underlined bases are inverted repeat).

Deletion of these repeats in a *Hin*dIII fragment abolished DNA replication in *Thermus*.

Any *Thermus* plasmid DNA, *Thermus* viral DNA, or genomic DNA can be digested with restriction enzymes to generate 2 - 20 kb fragments. The restriction fragments can be ligated with similarly-cut pUC-EKF or pUC-EKR and transformed into *Thermus* cells and selected for Km^R transformants. Alternatively, DNA can be extracted from

environmental samples, such as water from hot springs and soil sediment from hot springs, digested with restriction enzymes, ligated into similarly-cut pUC-EKF or pUC-EKR and transformed into *Thermus* cells and selected for Km^R transformants. Because of the small amount of DNA from environmental samples, one can transfer such DNA into *E. coli* first to amplify the DNA library and then transform such DNA into *Thermus*.

The following Examples are given to illustrate embodiments of the present invention, as it is presently preferred to practice. It will be understood that these Examples are illustrative, and that the invention is not to be considered as restricted thereto except as indicated in the appended claims.

The references cited above and below are herein incorporated by reference.

EXAMPLE I

1. Cloning of a replication origin from a *Thermus* plasmid pTsp45S native to *Thermus* species YS45.

Thermus species YS45 (Raven et al., *Nucl. Acids Res.* 21:4397 (1993) obtained from R.A.D. Williams of Queen Mary and Westerfield College, University of London) can be grown in modified *Thermus thermophilus* liquid media (Oshima and

Imahori, *J. Sys. Bacteriol.* 24:102-112 (1974)) consisting of 0.5% tryptone (DIFCO Laboratories; Detroit, Michigan), 0.4% yeast extract (DIFCO Laboratories; Detroit, Michigan), 0.2% NaCl at pH 7.5. Cells are plated in this media with 3% agar. Plated colonies are distinguishable after two days incubation at 55°-70°C. Individual colonies form dense liquid overnight cultures (3-10 ml) at 55°-70°C in a shaking waterbath. One-ml aliquots of overnight cultures are pelleted and stored at –20°C for up to one month without loss of viability. Overnight cultures are also stably maintained in media with 25% glycerol at -70°C.

Ten ml of 70°C overnight YS45 culture is diluted 1:1000 in 500 ml of *Thermus* media, and grown overnight at 70°C to generate plasmid DNA. Plasmid DNA is prepared via the Qiagen mid-prep protocol (Qiagen, Inc.; Studio City, California) with the addition of 2 mg lysozyme per ml. Lysis is very inefficient without the presence of lysozyme in the first resuspension buffer (Oshima and Imahori, *J. Sys. Bacteriol.* 24:102-112 (1974)). Routinely, between 50-150 μg of plasmid DNA is obtained from 500 ml of overnight YS45 culture.

YS45 contains two plasmids of 5.8 kb (pTsp45S) and approximately 12 kb (pTsp45L) (Wayne and Xu, *Gene* 195:321-328 (1997)). Each plasmid contains a single *Pst*I site useful for linearizing and visualizing the plasmids on agarose gels. Plasmid pTsp45S also contains two *Xba*I sites that generate

4.2 and 1.6-kb fragments. This plasmid is extensively mapped and cloned into pUC19 as three fragments: 4.2-kb *Xba*I-*Xba*I, 0.7-kb *Xba*I-*Pst*I, and 0.9-kb *Pst*I-*Xba*I. The 4.2-kb fragment is then further mapped and sub-cloned into pUC19 as six smaller fragments: 0.4-kb *Xba*I-*Hin*dIII, 1.1-kb *Hin*dIII-*Hin*dIII, 0.7-kb *Hin*dIII-*Hin*dIII, 0.5-kb *Hin*dIII-*Sca*I, 1.0-kb *Sca*I-*Sca*I, and 0.5-kb *Sca*I-*Xba*I. Cloning was accomplished by isolating digested fragments from agarose gels and combining them with compatibly cut pUC19 by standard methods (Sambrook et al., 'Molecular Cloning A Laboratory Manual', 2nd ed. (1989)).

The clones are sequenced using universal and reverse M13/pUC primers (New England Biolabs, Inc.; Beverly, Massachusetts). Preliminary sequencing was used to generate 12 additional primers (synthesized at New England Biolabs, Inc.; Beverly, Massachusetts) to refine and correct sequencing errors. The primers (shown as top and bottom strand pairs) are:

5'-GGTTCCATAAGGCGGGTCAATATAG-3' (SEQ ID NO:16);
5'-CTATATTGACCCGCCTTATGGAACC-3' (SEQ ID NO:17);
5'-GT GGGGTGGGCTGATCAAGAATCTCCT-3' (SEQ ID NO:18);
5'-AGGAGATTCTTGATCAGCCCACCCCAC-3' (SEQ ID NO:19);
5'-TCACCCACAACCCTCACGCACTCCAA-3' (SEQ ID NO:20);
5'-TTGGAGTGCGTGAGGGTTGTGGGTGA-3' (SEQ ID NO:21);
5'-AGATGTAGTCGTCCAGGGTGAGCCTG-3' (SEQ ID NO:22);
5'-CAGGCTCACCCTGGACGACTACATCT-3' (SEQ ID NO:23);

5'-TTGGTATGTAAAGCCCTTCGCGAGG-3' (SEQ ID NO:24);
5'-CCTCGCGAAGGGCTTTACATACCAA-3' (SEQ ID NO:25);
5'-TAGTGGCATCGGTGTTGTCGTGGGT-3' (SEQ ID NO:26); and
5'-ACCCACGACAACACCGATGCCACTA-3' (SEQ ID NO:27)

(underlined bases are in pTsp45s, but were not originally synthesized in these primers).

2. Characteristics of a thermophilic plasmid *ori*

The 2.3-kb *Nhe*I-bounded thermophilic *ori* is 57% G + C. The 5.8-kb *Thermus* plasmid pTsp45S is 54% G + C, and there are no other published reports of the G + C content in its natural host, YS45. There are no significant AT-rich regions within the sequenced *ori*.

The thermophilic *ori* contained one significant ORF of 1026 bp, beginning with GTG and ending with TGA (Figure 1). The ORF's 341 amino acid could encode a protein with a predicted molecular weight of 38.2 kDa. Centered 10 bp 5' of this ORF is a putative RBS, GGAGG (Hartmann and Erdmann, *J. Bacteriol.*, 171:2933-2941 (1989)). Further upstream, two possible promoter regions (-10 TATTTT, -35, TTGCCA, 17 bp spacing; or -10 TAGGGT, -35 TTGCCC, 18 bp spacing) were found (Figure 2) with significant homology to the *Thermus* consensus promoter (Maseda and Hoshino *FEMS Microbiol. Lett.* 128:127-134 (1985)). Database searches (FASTA, BLAST) did

not reveal any significant homologies to the predicted protein, or to other possible reading frames.

To test the importance of this ORF in the thermophilic replication, a significant portion of it was deleted. Briefly, pUC-EKF-Tsp3 was digested with *Nru*I + *Psh*AI, removing 234 bp or 78 aa within the ORF. The linearized plasmid was self-ligated, generating pUC-EKF-Tsp3-ΔNP(7.5 kb), then amplified in *E. coli* and used to transform HB27. No pUC-EKF-Tsp3-ΔNP(7.5 kb) Km^R transformants were found. It was concluded that 234 bp deletion within the *repT* gene abolished the replication function. Similarly, the addition of an *Xba*I amber stop linker (CTAGTCTAGACTAG (SEQ ID NO:28)) at either the *Nru*I or *Psh*AI site of pUC-EKF-Tsp3 negated thermophilic transformation. This indicated that the *repT* within the *Nhe*I fragment was necessary for replication in the thermophile. We suggest that this ORF of pTsp45S is a novel replication protein (RepT) needed for thermophilic plasmid replication. In addition, analysis of this thermophilic *ori* revealed two sequences with significant homology to highly conserved DnaA boxes. Although not yet described in *Thermus*, DnaA boxes are required for binding of a DnaA protein, and for subsequent replication of some plasmids (McMacken, et al., DNA Replication (Chapter 39), pages 586-587 in *Escherichia coli* and *Salmonella typhimmarium,* American Society for Microbiology, Washington, DC) . Both putative DnaA boxes (TTATCACCC (SEQ ID NO:29), TTATCCGAG (SEQ ID NO:30)) of pUC-EKF-Tsp3 lie within the 3' end of *repT*, and are not within

the region deleted in pUC-EKF-Tsp3-ΔNP. Plasmid copy number might be regulated by the relationship between binding of a DnaA homologue at these sites, and transcription of *repT*.

A sample of ER2688[pUC-EKF-Tsp3] has been deposited under the terms and conditions of the Budapest Treaty at the American Type Culture Collection on June 22, 1998, 1998 and received ATCC Accession No. 98793.

EXAMPLE II

Thermus YS45 strain contains two plasmids of 5.8 kb (pTsp45S) and approximately 12 kb (pTsp45L) (Wayne and Xu, *Gene* 195:321-328 (1997)). Each plasmid contains a single *Pst*I site useful for linearizing and visualizing the plasmids on agarose gels. The two plasmid mixture was digested with *Hin*dIII, *Kpn*I, *Pst*I, *Sph*I, or *Xba*I. The digested DNA fragments were cloned into pUC-EKR vector to produce *Thermus* DNA libraries and for subsequent selection of *Thermus* plasmid replication origin(s). Approximately 100, 100, 100, 100, and 50 Ap^R transformants were derived from pUC-EKR + *Hin*dIII fragments, + *Kpn*I fragments, + *Pst*I fragments, + *Sph*I fragments, and + *Xba*I fragments, respectively. Plasmids pUC-EKR with *Hin*dIII, *Kpn*I, *Pst*I, *Sph*I, or *Xba*I fragment inserts were amplified in *E. coli* and the DNA libraries were used to transform *Thermus thermophilus* HB27 (Pro^-). Transformants were plated on Km plates and incubated at 60°C for two days. Plasmid DNA was extracted from seventeen Km^R

transformants and digested with *Xba*I, *Pst*I, or *Sph*I. Restriction mapping and Southern blot analysis indicated that the 4.2 kb *Xba*I fragment *Thermus* origin insert was from pTsp45S, the 9 kb *Sph*I *Thermus* origin insert and the 12 kb *Thermus* origin insert were from pTsp45L. It was concluded that the entire pTsp45L plasmid can be separated into two *Sph*I fragments, 3 kb and 9 kb respectively. The 9 kb *Sph*I fragment contains the functional *Thermus* replication origin. The two *Sph*I fragments were sequenced by subcloning of one *Bam*HI fragment (1.4 kb), one *Hin*dIII fragment (1.9 kb), one *Sph*I fragment (3 kb), two *Kpn*I fragments (2.5 kb, 0.6 kb), three *Sac*I fragments (4.3 kb, 1.9 kb, 1.3 kb), and multiple *Sma*I fragments into pUC19. The inserts were sequenced by using pUC19 universal forward and reverse primers and by primer walking. Plasmid pTsp45L is 11958 bp, encoding 7 possible genes. These seven genes are named orf1 through orf7 (Figure 6). Orf1 amino acid sequence has weak similarity to transposases. Orf3 amino acid sequence has similarity to DNA replication protein RepA and DNA partition protein ParA. Orf4 amino acid sequence has similarity to serine carboxy peptidase III. Orf5 amino acid sequence has similarity to UvrB protein. Orf2, orf6, and orf7 amino acid sequences have no homologs to proteins in Genbank. The 3 kb *Sph*I fragment contains orf5 C-terminus portion, orf6 and orf7. Deletion of this 3 kb did not affect pTsp45L plasmid origin of replication. It was concluded that orfs 5, 6, and 7 are not required for plasmid replication. The 9 kb *Sph*I fragment contains the functional replication origin, which contains orf1, 2, 3, 4 and

a portion of orf5. Orf1 and orf4 have homology to transposases and proteases, respectively. It was concluded that orf1 and orf4 are unlikely involved in DNA replication and that orf3 is most likely the candidate for pTsp45L replication protein, because it has homolgy to RepA protein of *Agrobacterium* plasmid pTiB6S3, replication protein of *Agrobacterium* plasmid pRiA4b, plasmid partition protein of *Borrelia*, partition protein of *Frankia*, RepA protein of *Rhizobium*, and DNA partition protein ParA of *Caulobacter*. Orf2 may be an accessary protein for pTsp45L plasmid replication. Orf3 (coordinate 5876 to 6478) was renamed as *parA* gene. The DNA sequence and amino acid sequence of *parA* is shown in Figure 5. The location, direction, and organization of the seven open reading frames in pTsp45L are shown in Figure 6.

There are direct repeats and inverted repeats in the 9 kb *Sph*I fragment containing the functional replication origin. The direct repeats I are:

5' GGCTTTTCTT 3' (SEQ ID NO:9)
5' AACTTTTCCC 3' (SEQ ID NO:10)
5' GACTTTTTTC 3' (SEQ ID NO:11)
consensus 5' RRCTTTTYYY 3' (SEQ ID NO:1)

The direct repeats II are:

5' AACTTTG 3' (SEQ ID NO:12)
5' AGTTTTG 3' (SEQ ID NO:13)
5' GATTTTG 3' :(SEQ ID NO:14)
5' AACTTTG 3' (SEQ ID NO:15)
consensus 5' RRYTTTG 3' :(SEQ ID NO:2)

The inverted repeat is:

5' TTAACCTTTTTTCAAGAAAAAGAGATAA 3' (SEQ ID NO:3)
3' AATTGGAAAAAAGTT CTTTTTCTCTATT 5'
(COMPLEMENT OF SEQ ID NO:3)

(underlined bases are inverted repeats).

The repeats and inverted repeats are important for pTsp45L origin of replication, because deletion of these repeats in a *Hin*dIII fragment abolished DNA replication in *Thermus*. The DNA sequence of pTsp45L is shown in Figure 7. The *Thermus-E. coli* shuttle vector containing pTsp45L DNA replication origin was named as pUC-EKR-Tsp45L9Kb.

A sample of ER2688[pUC-EKR-Tsp45L9kb] has been deposited under the terms and conditions of the Budapest Treaty at the American Type Culture Collection on June 22, 1998, and received ATCC Accession No. 98794.

EXAMPLE III

Thermus strain YS45 (Raven, et al., *Nucl. Acids Res.* 21:4397 (1993) obtained from R.A.D. Williams of Queen Mary and Westerfield College, University of London) also harbors a plasmid. Plasmid DNA was extracted from *Thermus* species YS45 by midi Qiagen column. The plasmid DNA was cleaved with *Hin*dIII, *Kpn*I, *Pst*I, *Sph*I, or *Xba*I. The digested DNA fragments were cloned into pUC-EKR vector to produce *Thermus* DNA libraries and for subsequent selection of *Thermus* plasmid replication origin(s). Approximately 50 to

300 Ap^R *E. coli* transformants were derived from pUC-EKR + *Hin*dIII fragments, + *Kpn*I fragments, + *Pst*I fragments, + *Sph*I fragments, and + *Xba*I fragments, respectively. Plasmids pUC-EKR with *Hin*dIII, *Kpn*I, *Pst*I, *Sph*I, and *Xba*I fragment inserts were amplified in *E. coli* and the DNA libraries were used to transform *Thermus thermophilus* HB27 (Pro^-). Transformants were plated on Km plates and incubated at 60°C for two days. *Thermus* transformants were found in *Hin*dIII and *Pst*I DNA libraries. Plasmid DNA was extracted from seventeen Km^R *Thermus* transformants and digested with *Hin*dIII or *Pst*I. It was found that the functional Tse plasmid replication origin was contained in a ~7 kb *Hin*dIII or *Pst*I fragment. The shuttle vector was named pUC-EKR-Tse7Kb.

EXAMPLE IV

Thermus cells can be grown in modified *Thermus thermophilus* liquid media (Oshima and Imahori, *J. Sys. Bacteriol.* 24:102-112 (1974)) consisting of 0.5% tryptone (DIFCO Laboratories; Detroit, Michigan), 0.4% yeast extract (DIFCO Laboratories; Detroit, Michigan), 0.2% NaCl at pH 7.5. *Thermus* cells can also be cultured in 4 to 10-fold diluted rich both at 50°-75°C. Ten ml of overnight cell culture is diluted 1:1000 in 500 ml of *Thermus* media, and grown overnight at 50°-75°C to generate plasmid DNA. Plasmid DNA can be prepared via the Qiagen midi/maxi-prep protocol (Qiagen, Inc.; Studi City, California) with the addition of 2 mg lysozyme per ml or any other plasmid preparation method such as alkaline

lysis or boiling methods. The purified plasmid DNA can be digested with restriction enzymes to produce DNA fragments of 2 to 20 kb. The plasmid DNA can also be sonicated to produce blunt end framgents and be made into sticky ends by addition of deoxynucleotides by terminal nucleotide transferase. The DNA fragments can be cloned into pUC-EKF or pUC19-EKR and the ligated DNA can be used for thermophilic transformation into *Thermus* cells. Transformants can be selected by plating cells on Km plates. Any Km^R transformants should contain *Thermus* plasmid replication origin. The origin can be further subcloned and sequenced. A minimal replication origin can be defined by subcloning smaller DNA fragments into pUC-EKF or pUC19-EKR and the resulting plasmid DNA can be used for thermophilic transformation.

Alternatively, plasmid DNA, *Thermus* viral DNA or genomic DNA can be extracted from environmental samples such as water from hot springs and soil sediment from hot springs and digested with restriction enzymes and ligated into similarly-cut pUC-EKF or pUC-EKR. The ligated DNA can be transformed into *Thermus* cells and select for Km^R transformants. Because of the small amount of DNA from environment samples, one can transfer DNA into *E. coli* first to amplify DNA library and then transform into *Thermus*. The thermophilic replication origin can be further subcloned and sequenced. A minimal replication origin can defined by subcloning smaller DNA fragments into pUC-EKF or pUC19-EKR

and the resulting plasmid DNA can be used for thermophilic transformation.

WHAT IS CLAIMED IS:

1. An isolated DNA encoding a *Thermus sp.* plasmid replication protein, said isolated DNA comprising the sequence of SEQ ID NO:4 or conservatively modified variants thereof.

2. A recombinant plasmid comprising at least one *Thermus sp.* replication origin, wherein said replication origin includes the isolated DNA sequence of claim 1.

3. The recombinant plasmid of claim 2, further comprising at least one promoter sequence selected from the group consisting of the DNA sequence of SEQ ID NO:6, residues 27-32 of SEQ ID NO:6, residues 50-55 of SEQ ID NO:6, residues 86-90 of SEQ ID NO:6, and residues 109-114 of SEQ ID NO:6.

4. An *E. coli sp.* host cell transformed with the recombinant plasmid of claims 2 or 3.

5. A *Thermus sp.* host cell transformed with the recombinant plasmid of claims 2 or 3.

6. An isolated DNA encoding a *Thermus sp.* promoter, wherein said isolated DNA is selected from the group consisting of the DNA sequence of SEQ ID NO:6, residues 27-32 of SEQ ID NO:6, residues 50-55 of SEQ ID NO:6, residues 86-90 of SEQ ID NO:6, and residues 109-114 of SEQ ID NO:6.

7. An isolated DNA encoding a *Thermus sp.* plasmid replication protein, said isolated DNA comprising the sequence of SEQ ID NO: 7 or conservatively modified variants thereof.

8. A recombinant plasmid comprising the isolated DNA sequence of claim 7 and a functional replication origin comprising the DNA sequences of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, and SEQ ID NO:15 and the complements of the DNA sequences of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, and SEQ ID NO:15.

9. An *E. coli sp.* host cell transformed with the recombinant plasmid of claim 8.

10. A *Thermus sp.* host cell transformed with the recombinant plasmid of claim 8.

11. A method for cloning *Thermus sp.* plasmid genes comprising the steps of:

(a) isolating plasmid DNA from *Thermus sp.* cells;

(b) inserting said plasmid DNA into a recombinant plasmid comprising a thermostable kanomycin-resistant gene and an *E. coli* replication origin;

(c) transforming an *E. coli sp.* host cell with the recombinant plasmid of step (b) and culturing said *E. coli sp.*

host cell under conditions suitable for the expression of said recombinant plasmid;

(d) isolating cloned recombinant plasmid from said cells; and

(e) transforming a *Thermus sp.* host cell with said cloned recombinant plasmid from step(d) and culturing said *Thermus sp.* host cell under conditions suitable for the expression of said recombinant plasmid.

ABSTRACT

The present invention relates to cloned DNA containing origin of DNA replication and to cloned DNA encoding repliation protein, RepT.

Fig. 1

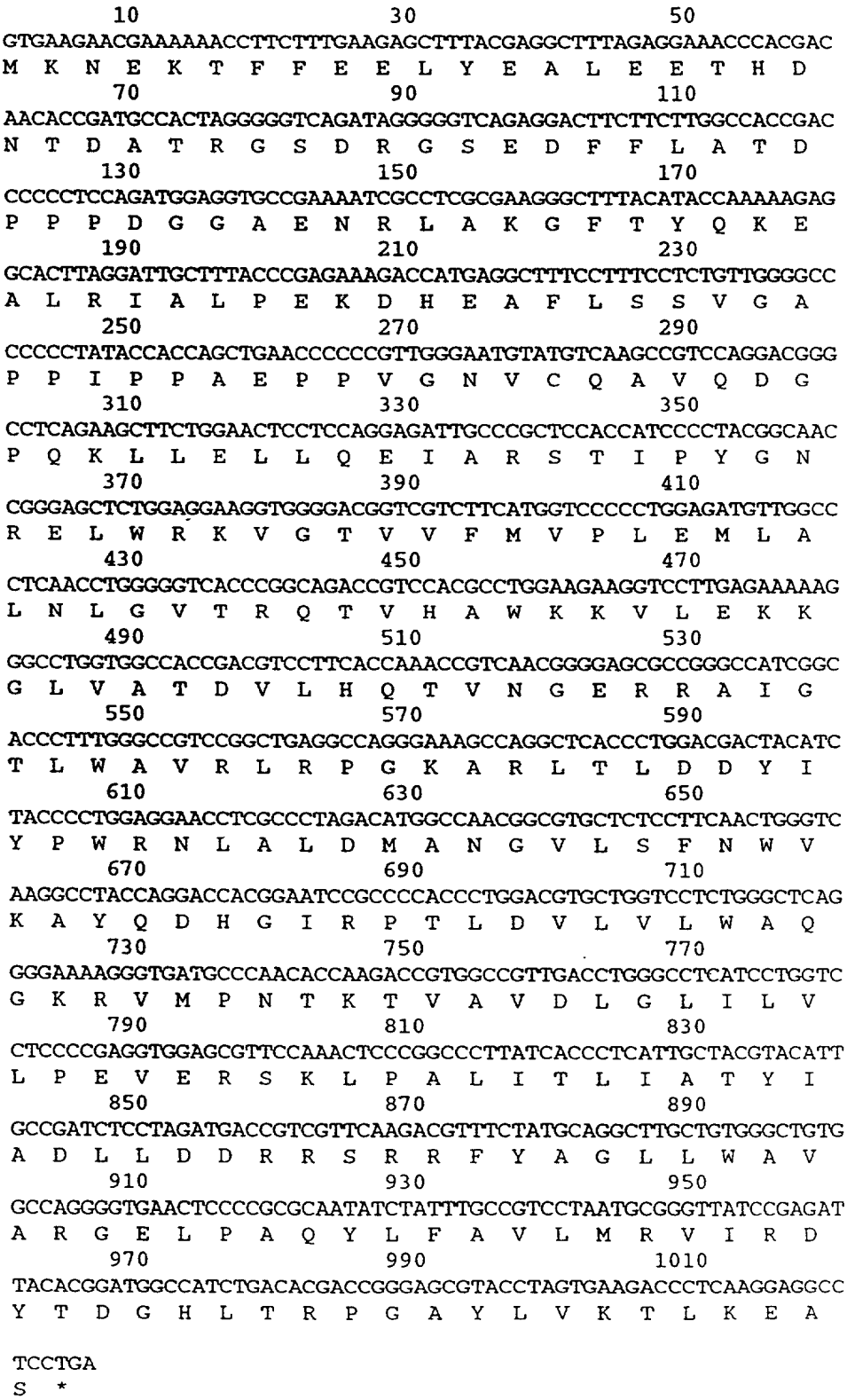
10 30 50
GTGAAGAACGAAAAAACCTTCTTTGAAGAGCTTTACGAGGCTTTAGAGGAAACCCACGAC
M K N E K T F F E E L Y E A L E E T H D
70 90 110
AACACCGATGCCACTAGGGGGTCAGATAGGGGGTCAGAGGACTTCTTCTTGGCCACCGAC
N T D A T R G S D R G S E D F F L A T D
130 150 170
CCCCCTCCAGATGGAGGTGCCGAAAATCGCCTCGCGAAGGGCTTTACATACCAAAAAGAG
P P P D G G A E N R L A K G F T Y Q K E
190 210 230
GCACTTAGGATTGCTTTACCCGAGAAAGACCATGAGGCTTTCCTTTCCTCTGTTGGGGCC
A L R I A L P E K D H E A F L S S V G A
250 270 290
CCCCCTATACCACCAGCTGAACCCCCCGTTGGGAATGTATGTCAAGCCGTCCAGGACGGG
P P I P P A E P P V G N V C Q A V Q D G
310 330 350
CCTCAGAAGCTTCTGGAACTCCTCCAGGAGATTGCCCGCTCCACCATCCCCTACGGCAAC
P Q K L L E L L Q E I A R S T I P Y G N
370 390 410
CGGGAGCTCTGGAGGAAGGTGGGGACGGTCGTCTTCATGGTCCCCCTGGAGATGTTGGCC
R E L W R K V G T V V F M V P L E M L A
430 450 470
CTCAACCTGGGGGTCACCCGGCAGACCGTCCACGCCTGGAAGAAGGTCCTTGAGAAAAAG
L N L G V T R Q T V H A W K K V L E K K
490 510 530
GGCCTGGTGGCCACCGACGTCCTTCACCAAACCGTCAACGGGGAGCGCCGGGCCATCGGC
G L V A T D V L H Q T V N G E R R A I G
550 570 590
ACCCTTTGGGCCGTCCGGCTGAGGCCAGGGAAAGCCAGGCTCACCCTGGACGACTACATC
T L W A V R L R P G K A R L T L D D Y I
610 630 650
TACCCCTGGAGGAACCTCGCCCTAGACATGGCCAACGGCGTGCTCTCCTTCAACTGGGTC
Y P W R N L A L D M A N G V L S F N W V
670 690 710
AAGGCCTACCAGGACCACGGAATCCGCCCCACCCTGGACGTGCTGGTCCTCTGGGCTCAG
K A Y Q D H G I R P T L D V L V L W A Q
730 750 770
GGGAAAAGGGTGATGCCCAACACCAAGACCGTGGCCGTTGACCTGGGCCTCATCCTGGTC
G K R V M P N T K T V A V D L G L I L V
790 810 830
CTCCCCGAGGTGGAGCGTTCCAAACTCCCGGCCCTTATCACCCTCATTGCTACGTACATT
L P E V E R S K L P A L I T L I A T Y I
850 870 890
GCCGATCTCCTAGATGACCGTCGTTCAAGACGTTTCTATGCAGGCTTGCTGTGGGCTGTG
A D L L D D R R S R R F Y A G L L W A V
910 930 950
GCCAGGGGTGAACTCCCCGCGCAATATCTATTTGCCGTCCTAATGCGGGTTATCCGAGAT
A R G E L P A Q Y L F A V L M R V I R D
970 990 1010
TACACGGATGGCCATCTGACACGACCGGGAGCGTACCTAGTGAAGACCCTCAAGGAGGCC
Y T D G H L T R P G A Y L V K T L K E A
TCCTGA
S *

Fig. 2

1 CTATAACGGCCTTTTAGGAGGGGGGATTGCCAGCCGCTGGGCTGACGGTTATTTTGGACC

61 CATAAAAAGGCGAAACCGAGGCGGTTGCCCCGGATCACCCCCAAGACCTAGGGTAACGCC

121 TCGGGCTCCAGATGACAA*GGAGG*TCCGAGGGTGAAGAACGAAAAAACCTTCTTTGAAGAG

M K N E K T F F...(RepT)

Fig. 3

```
   1 tctagaaggt cagggtggac aaggaaaaca ccatagcccc tgccaagaag atggacgagt
  61 tggtgtccgg aaaagtggcc atccggggcg ctcttgacaa ctattttcca gcggtggcca
 121 ccggcattgg ccacgaggta cgagcttgtg gagtagacgg ccacaaaggg gtcgtcctca
 181 aacttctttt ctagtgccgc ttggacgaag gggaggaaga ggaaaggctt catggcctca
 241 cctccttccc ctcctccttg gcggccttag cggcgtaaaa ctctgagacg gcctgaagtt
 301 tagggatttc gctttcgggg ataagaatcc ggcggctcag gggatgccgg atggccctta
 361 tcctgccgtc ccttatgtac tcgtaaatgg tggccttggg tactttaaac cgttctgaaa
 421 cttctctaac agagagcaca aaacctctaa aaacctatca atcccaccga ttccagtata
 481 ccataaatgg cacaaagttt tgagaaggtg gtcaaacaaa aaggctttct cggtcaggtt
 541 atggtgaggt gggggcggtc aaaggccgac ttaagtttgg taaagccggg aggaagcaaa
 601 ccggggtgtt accatgcaac agatggccga gtggaacgtg tggacacaga gaagcgttga
 661 gcttctggag aaggggtatt tggataaact actgcaggtc tataaagggg aaagtggctc
 721 ttcgaggtca gtaccagagg aggtagagga aaaacttcgc gaggcctaca aggcatacga
 781 ggggaggcag gatagtccgg aggcagaaac gaaactcgtg gaagccgtgc taaatgccag
 841 aaaaaaggtc gagcggtccc ccttcaatca cccctacctg cctttggtct actacctggt
 901 ttcggaaaaa gcagaaaaag cgaacaaggc ccttgaggag gcattgcagg aggttgcctc
 961 aaagcaccca gaaaccatcc gcgtcctggc caaggaagcg caaagaagag gcgtagaagc
1021 cttgatccaa aggctcaagg agcctcccga aataaatcgg cagatagggc cgatgttcaa
1081 aaggtggtac aaagaagagc taaaggggaa aatagaagag aggcttccag gccctaccaa
1141 accaaagatt gtggtagtat cccctgaaaa aagtaaaccg gagcaagcac cccttattgc
1201 ggagagagaa gcgggcatca tcatatacac gggatcggat gaagctttga aagatgccgc
1261 caaggaaaac ctgggccttg gcgaggaagc agaactaggc accaagggcg tagatttcta
1321 cgtggtcatc cggcgtagcc ctgaagagac atggcaccta acaggagaag tgaagtttca
1381 atccgacttt ggcggaaacc aagacaacca gaaactagta gcaaaggctt ccataaggtt
1441 ggaccttgag aagaggcaca taggaatagt ggtggtggac ggaatgcctg tggtgagcaa
1501 gtttcgtggg tgggccggac tggggaaaga aacgatcgtt acatccgtac tcctccttcc
1561 agacctgata gcggagctct accaaaaggg tgaagaagcc ctgggcctct agaaggcgga
1621 cacaatctca aacttgtgct gtagcctggg gaaatcctct aacacccttc tagtgaaggc
1681 tttgaccgcc tcccaggagg catctatgcc gatggatcgc cgctttaaga ggggtgaggc
1741 tataagcgta gtaccggagc ctgcgaaggg atcgagcact aaatcccccc cgttactccc
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1921 gtaaactttc ttccgcggca ccccgttctt tgaccagaca ataagccctt gagcgtctag
1981 ctcgtcaagc ttctccgggg gatagcgcca atgccgtcca ggaggggaa gtattcctcg
2041 ccaaggcctt ccggtagggc catccttggt ttctccagga gcatgcaggg gattggtggt
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2161 ggggctagcc gattcgttcc aaacgtagtc ccgcgttttg gagtagacga ggatcatgtc
2221 cttttgcgat ccgaaggcct tacgggaaaa gtttttggga tttgaagcga tgcgggcgat
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2341 gtatttctcg tctatgtgaa cgaagatcag tcctgagtcc gccatcagct ccctgagaag
2401 tatcaagcgc tccctcagga actccacaaa ctgaggacca tcgagggtgt catcgtagcc
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2641 ggtgccaata ggatcaatct caaaaagggg ggcatttccc cctaggaaga ggagggtttc
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2761 ggggtagacc aacctaaagg gcgaaggttc cgaggttttc gaggctttca agggggcttt
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Fig. 3 (continued)

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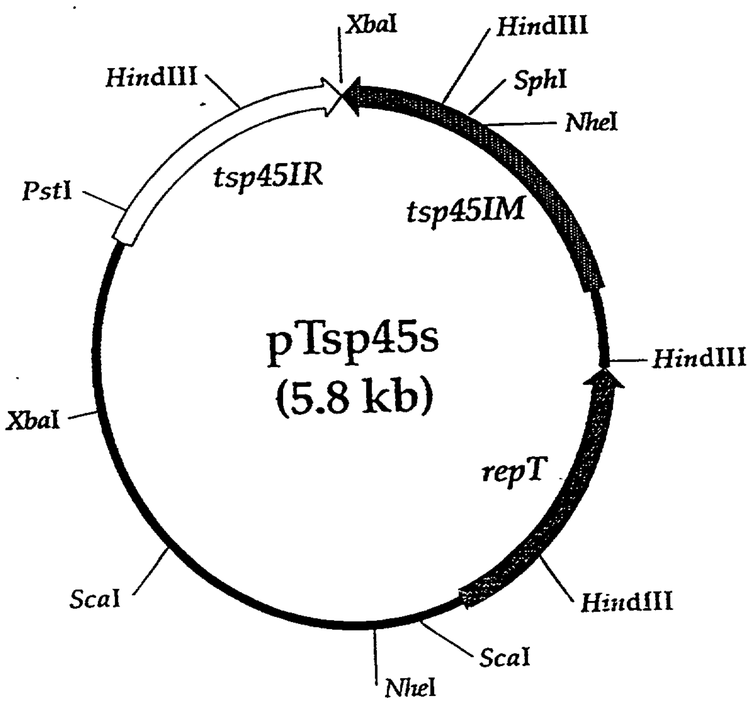
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ScaI
NheI

Fig. 4

Fig. 5

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     L  A  C  F  L  A  E  R  G  P  T  L  L  V  D  G  D  P  N  R
121  TCCGCCACGGGGTGGCACCGGAGGGGAGGCCTCCCGGTGACCGTGGTGGACGAGCGGGTG
     S  A  T  G  W  H  R  R  G  G  L  P  V  T  V  V  D  E  R  V
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     A  A  R  Y  A  R  E  H  A  H  V  V  I  D  T  Q  A  R  P  T
241  GAAGAGGACCTCCGGGCCCTCGCCAAGGGGGTGGACCTGCTGGTCCTGCCCACGTCCCCC
     E  E  D  L  R  A  L  A  K  G  V  D  L  L  V  L  P  T  S  P
301  GACGCCCTGGCCCTGGAGGCCCTCCTGGCCACCCTGGAAGCCCTGCGGGGGGCGGAGGCC
     D  A  L  A  L  E  A  L  L  A  T  L  E  A  L  R  G  A  E  A
361  CGCTTCCGGGTCCTCCTGACCATGGTGCCCCCGCCCCCGAGCCGGGACGGGGAGGAGGCC
     R  F  R  V  L  L  T  M  V  P  P  P  P  S  R  D  G  E  E  A
421  CGGGCCCTCTTGGGGGCGGAGGGCGTTCCCCTCTTCACAGGCTGGGTGAGGCGGGCGGCA
     R  A  L  L  G  A  E  G  V  P  L  F  T  G  W  V  R  R  A  A
481  GCCTTCCCCAAGGCCGCCCTCCTGGGGGTGCCTGTCTACCGGGTGCCCGACCCCAGGGCG
     A  F  P  K  A  A  L  L  G  V  P  V  Y  R  V  P  D  P  R  A
541  AGGCTGGCCTGGGGGGACTACGCGCGGGTGGGGGAAGAGCTCCTGAAGGAGGTGGGGGA
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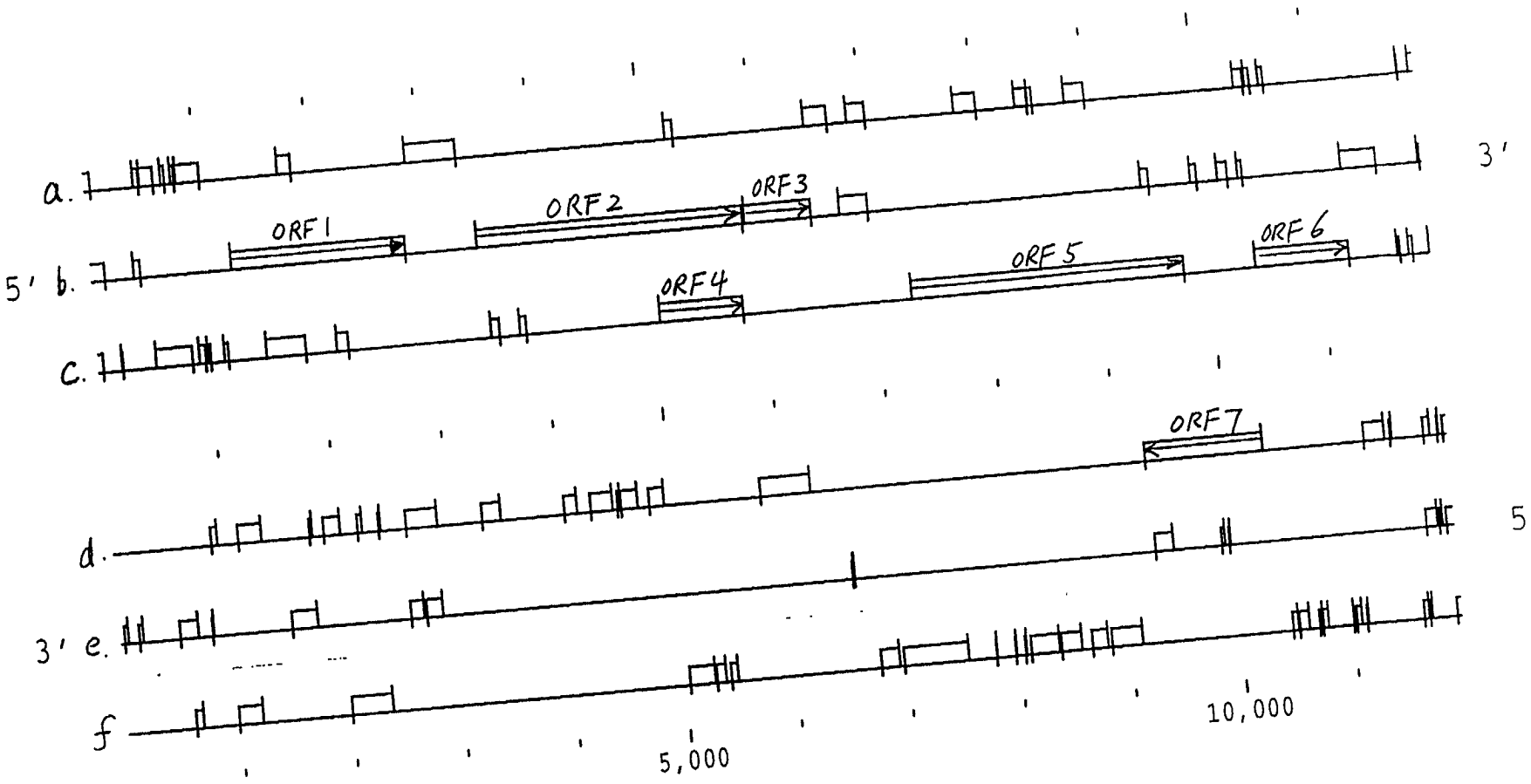
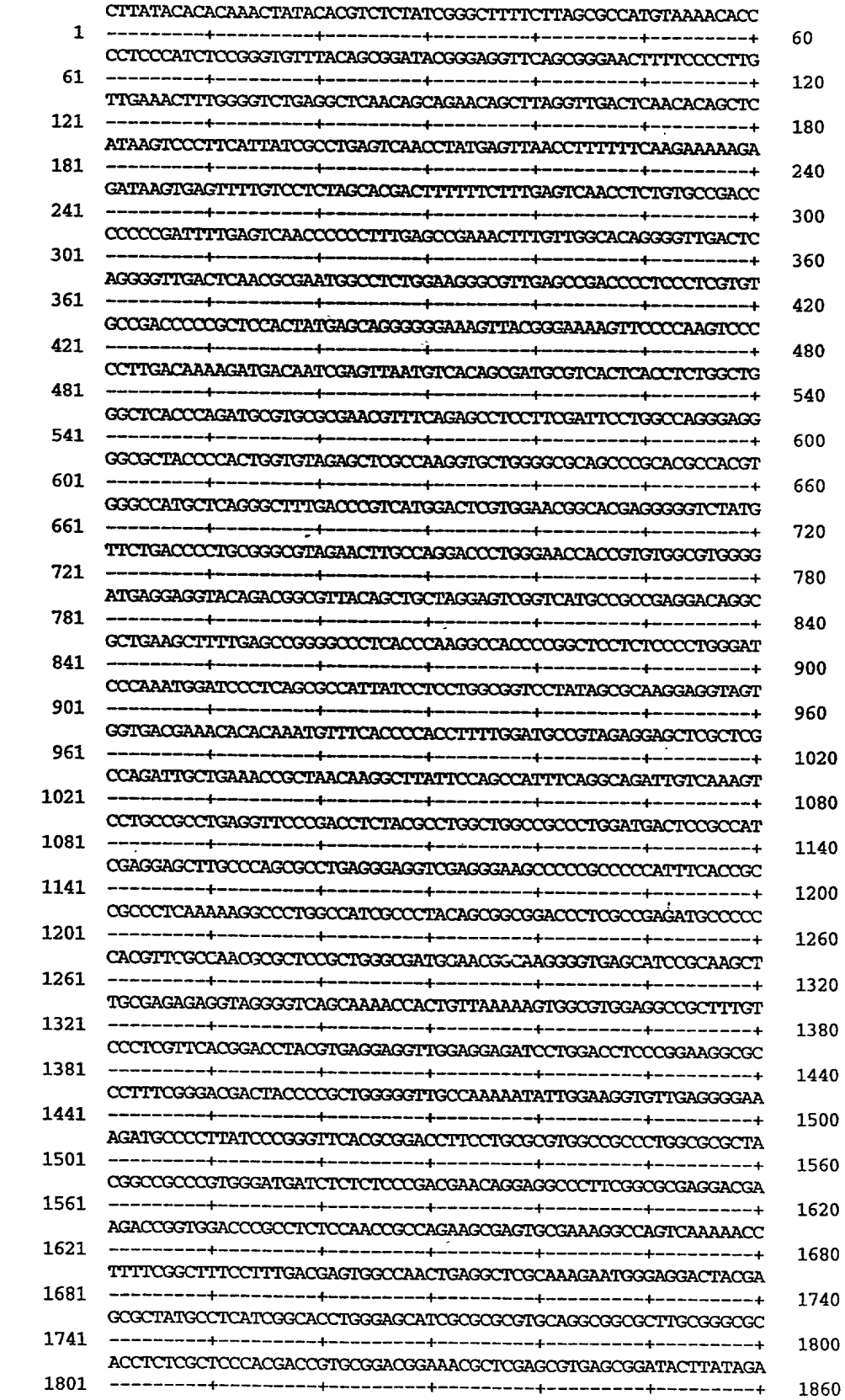


Fig. 6

Fig. 7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121 180
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181 240
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661 720
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721 780
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781 840
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841 900
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901 960
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961 1020
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1081 1140
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Fig. 7 (continued)

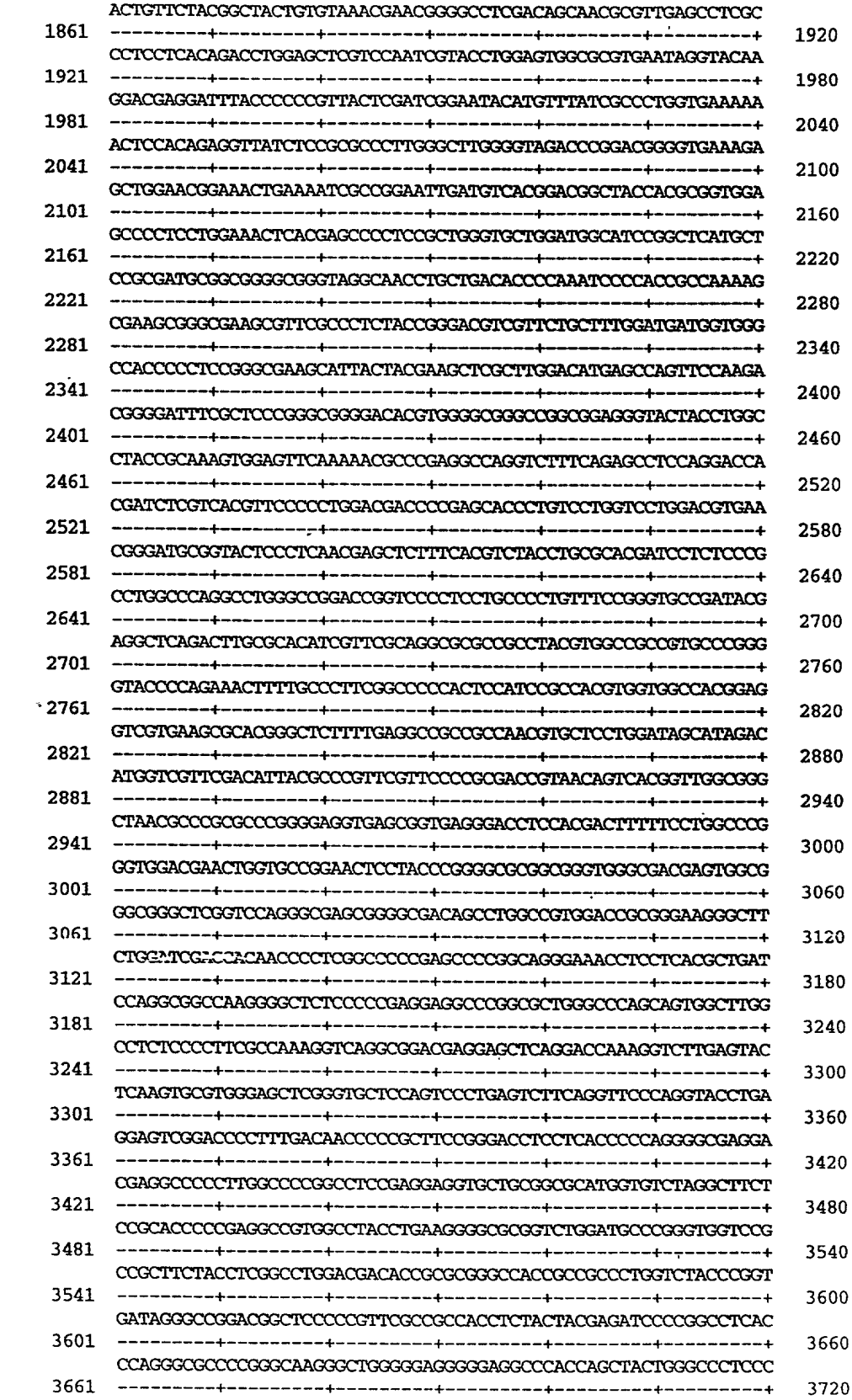

Fig. 7 (continued)

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3781  ---------+---------+---------+---------+---------+---------+  3840
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3841  ---------+---------+---------+---------+---------+---------+  3900
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3901  ---------+---------+---------+---------+---------+---------+  3960
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3961  ---------+---------+---------+---------+---------+---------+  4020
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4081  ---------+---------+---------+---------+---------+---------+  4140
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4141  ---------+---------+---------+---------+---------+---------+  4200
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4201  ---------+---------+---------+---------+---------+---------+  4260
      CCTGGGCTGGGGCTACTTGCCGGCCCCGCCCGGCACCCCCTTGGAGGACCGGGTGCTGGC
4261  ---------+---------+---------+---------+---------+---------+  4320
      CGTGGACGACGGCACCATCATCCGCAGGCCCCCGAAGGCGGCCGCCGGGACCTCGTGGAA
4321  ---------+---------+---------+---------+---------+---------+  4380
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4381  ---------+---------+---------+---------+---------+---------+  4440
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4501  ---------+---------+---------+---------+---------+---------+  4560
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4561  ---------+---------+---------+---------+---------+---------+  4620
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4621  ---------+---------+---------+---------+---------+---------+  4680
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4801  ---------+---------+---------+---------+---------+---------+  4860
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5401  ---------+---------+---------+---------+---------+---------+  5460
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5461  ---------+---------+---------+---------+---------+---------+  5520
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Fig. 7 (continued)

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5641 ---------+---------+---------+---------+---------+---------+ 5700
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5701 ---------+---------+---------+---------+---------+---------+ 5760
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5761 ---------+---------+---------+---------+---------+---------+ 5820
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5881 ---------+---------+---------+---------+---------+---------+ 5940
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6241 ---------+---------+---------+---------+---------+---------+ 6300
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6301 ---------+---------+---------+---------+---------+---------+ 6360
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6361 ---------+---------+---------+---------+---------+---------+ 6420
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6421 ---------+---------+---------+---------+---------+---------+ 6480
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7021 ---------+---------+---------+---------+---------+---------+ 7080
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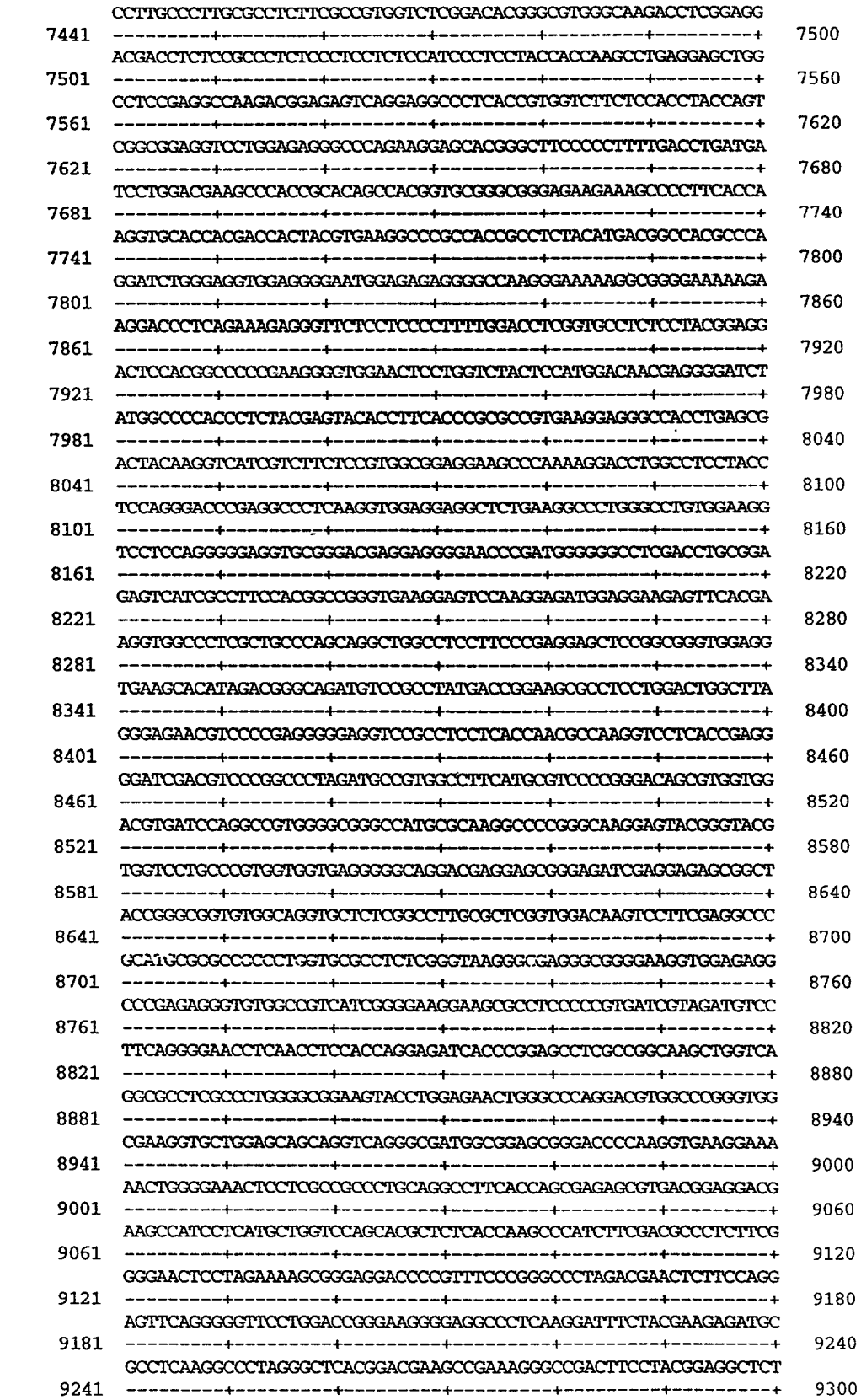
Fig. 7 (continued)



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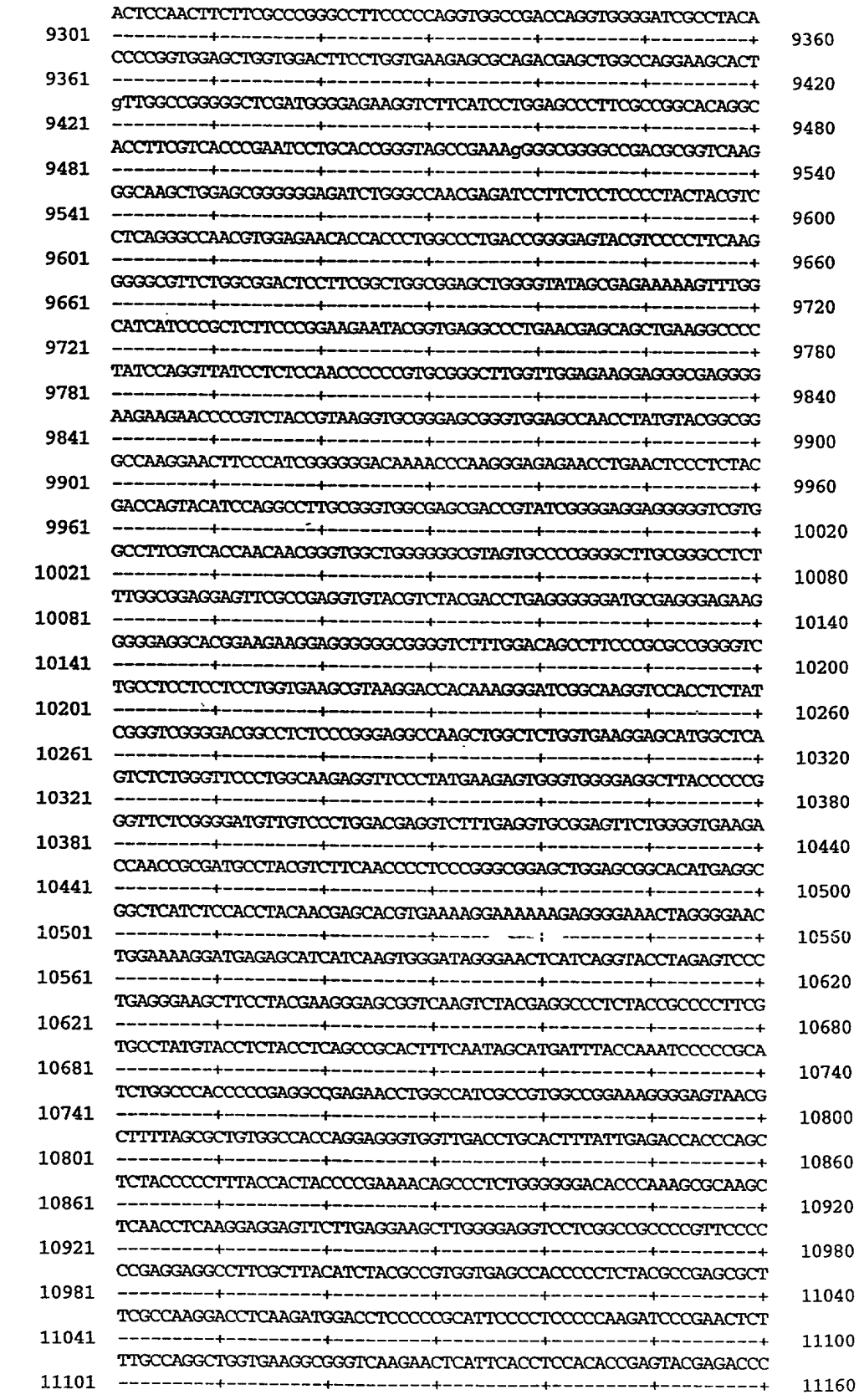

Fig. 7 (continued)

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11221 ---------+---------+---------+---------+---------+---------+ 11280
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New England Biolabs, Inc.
32 Tozer Road
Beverly, MA 01915

DECLARATION
AND POWER OF ATTORNEY
Original Application

Attorney Docket No. NEB-135

COPY

As a below named inventor, I hereby declare that:
My residence, post address and citizenship are as stated below next to my name
I believe that I am the original, first and sole inventor (in only one name is listed at 201 below) or an original, first and joint inventor (if plural names are listed at 201-203 below) of the subject matter which is claimed and which a patent is sought on the invention entitled:

METHOD FOR CONSTRUCTION OF THERMUS-E. COLI SHUTTLE VECTORS AND IDENTIFICATION OF TWO THERMUS PLASMID REPLICATION ORIGINS

which is described and claimed in:

[ ] the attached specification or [X] the specification in Application Serial No. 09/134,246 filed 8/14/98
(for declaration not accompanying application)
And was amended on _____
if applicable

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendments referred to above. I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, §1.56(a). I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

FOREIGN APPLICATION(S) IF ANY, FILED WITHIN 12 MONTHS PRIOR TO THE FILING DATE OF THIS APPLICATION			
COUNTRY	APPLICATION	DATE OF FILING (day, month, year)	PRIORITY CLAIMED UNDER 35 U.S.C. 119
			YES NO
			YES NO
ALL FOREIGN APPLICATION(S) IF ANY, FILED MORE THAN 12 MONTHS PRIOR TO THE FILING DATE OF THIS APPLICATION			
COUNTRY	APPLICATION	(day, month, year)	PRIORITY CLAIMED UNDER 35 U.S.C. 119

I hereby claim the benefit under Title 35, United States Code §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

Application Serial No.	Filing Date	Status (Patented, Pending, Abandoned)

DECLARATION
AND POWER OF ATTORNEY

POWER OF ATTORNEY:

As a named inventor, I hereby appoint the following attorney with full powers of association, substitution and revocation to prosecute this application and transact all business in the Patent and Trademark Office connected therewith:

Gregory D. Williams
(Registration No. 30901)

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Gregory D. Williams
General Counsel
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Gregory D. Williams
General Counsel
New England Biolabs, Inc.
Tele: (508) 927-5054; Ext. 292
Fax: (508) 927-1705

2	Full Name of Inventor	Last Name Wayne	First Name Jay	Middle Name
0	Residence & Citizenship	City Flushing	State/Foreign Country New York	Citizenship US
1	Post Office Address	Post Office Address 142-25 Horace Harding Expressway	City/State/Country Flushing, New York	Zip Code 11367
2	Full Name of Inventor	Last Name Xu	First Name Shuang-yong	Middle Name
0	Residence & Citizenship	City Lexington	State/Foreign Country Massachusetts	Citizenship China
2	Post Office Address	Post Office Address 30 Grapevine Avenue	City/State/Country Lexington, Massachusetts	Zip Code 02173
2	Full Name of Inventor	Last Name	First Name	Middle Name
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DECLARATION
AND POWER OF ATTORNEY
PAGE 3 OF 3

206	Full Name of Inventor	Last Name	First Name	Middle Name
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208	Full Name of Inventor	Last Name	First Name	Middle Name
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209	Full Name of Inventor	Last Name	First Name	Middle Name
	Residence & Citizenship	City	State/Foreign Country	Citizenship
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I hereby further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true and further that these statements were made with the knowledge that willful statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Signature of Inventor 201 Jay Wayne	Date 8/24/98
Signature of Inventor 202	Date Sept. 16 1998
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Xu, Shuang-yong

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